Vitamin D analogs
in experimental leukemia and
transplantation models

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To my dear wife Anna

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I Pakkala I, Savli H, Knuutila S, Binderup L, Pakkala S. CB1093, a novel vitamin D analog; effects on differentiation and clonal growth on HL-60 and de novo leukemia cells. Leukemia Research 1997; 21: 321-326

II Pakkala I, Savli H, Räisänen-Sokolowski A, Pakkala S. Vitamin D analogs EB1089 and CB1093 combined with idarubicin inhibited proliferation and promoted apoptosis in HL-60 leukemia cells. Submitted.

III Räisänen-Sokolowski A, Pakkala I, Samila S, Binderup L, Häyry P, Pakkala S. A vitamin D analogue, MC1288, inhibits adventitial inflammation and suppresses intimal lesions in rat aortic allografts. Transplantation 1997; 63, 936-941

IV Pakkala I, Taskinen E, Pakkala S, Räisänen-Sokolowski A. MC1288, A vitamin D analog, prevents acute graft-versus-host disease in rat bone marrow transplantation. Bone Marrow Transplantation 2001; 27, 863-867

ABBREVIATIONS

aGVHD,	acute graft-versus-host disease
AML,	acute myeloid leukemia
APC,	antigen presenting cell
APL,	acute promyelocytic leukemia
BMT,	bone marrow transplantation
BSA,	bovine serum albumin
CB1093,	1(S), 3(R)-Dihydroxy-20(R)-(1(S)-1-ethoxy-5-ethyl-5-hydroxy-2-heptyn-1-yl)-9,10-seco-pregna-5(Z), 7(E), 10(19)-triene
Cdk,	cyclin-dependent kinase
CR,	complete remission
CsA,	cyclosporin A
DLI,	donor lymphocyte infusion
EAE,	murine experimental autoimmune encephalomyelitis
EB1089,	24a,26a,27a-tri-homo-22,24-diene-1-alfa,25-$(OH)_2$-D_3
FK506,	tacrolimus
GVHD,	graft-versus-host disease
HLA,	human leukocyte antigen
HSCT,	hematopoietic stem cell transplantation
IL-2,	interleukin-2
MC1288,	20-epi-1,25$(OH)_2D_3$
MLC,	mixed lymphocyte culture
MMF,	mycophenolate mofetil
NK cell,	natural killer cell
PHA,	phytohemagglutin
RA,	retinoic acid
RAR,	retinoic acid receptor
RARE,	retinoic acid-responsive element
RXR,	retinoid X receptor
SCT,	stem cell transplantation
TCR,	T cell receptor
TNF,	tumor necrosis factor
VD_3,	$1\alpha25(OH)_2D_3$, the active form of vitamin D_3
VDR,	vitamin D_3 receptor
VDRE,	vitamin D_3-responsive element

INTRODUCTION

In the treatment of acute leukemia there are two main problems. After chemotherapy, the long term results in maintenance therapy have not improved. Another way to treat acute leukemia is stem cell transplantation in which acute graft-versus-host disease is a major cause of mortality. Novel vitamin D analogs have demonstrated both antileukemic and immunosuppressive properties. This provides possible alternatives to current practice.

A differentiation therapy suitable for clinical use would decisively change the treatment practice of acute myeloid leukemia. This has already happened in the treatment of acute promyelocytic leukemia where patients can achieve remission using retinoic acid (RA) without the side effects of chemotherapy.

Stem cell transplantation is the common practice in leukemia for patients with a suitable donor. Main problems arise from acute graft-versus-host disease (aGVHD), which necessitates long treatment periods and high dose use of steroids. Due to lack of suitable related donors the use of unrelated, HLA-matched donors from international marrow-donor registries has increased. This has lead to a better prognosis in the treatment of leukemia itself, due to graft-versus-leukemia effect, but with the cost of higher incidence of GVHD.

In solid organ transplantation many transplants are lost due to chronic rejection (transplant arteriosclerosis). The medication used for chronic rejection today has marked side effects that severely limit the immunosuppressive effect that can be induced with these drugs. We need to develop new immunosuppressive strategies that minimize side effects but still are potential in preventing graft rejection.

REVIEW OF THE LITERATURE

1. Background

1.1 Acute myeloid leukemia

The hematopoietic system produces blood cells by committing required stem cells into maturation. These committed cells lose their ability to divide while they mature into functional blood cells. The stem cells are self-renewable and normally preserved in a non-cycling state.

The prominent abnormality of acute myeloid leukemia (AML) is the inability of the cells to differentiate to functional mature cells. The maturation of the cells is blocked at an early stage and the cells remain in a rapidly dividing proliferative pool. Thus, AML is characterized by the proliferation of abnormal leukemic blast cells, and impaired production of normal blood cells, leaving the patient prone to anemia, thrombocytopenia, neutropenia, and bleeding as well as infection.

Treatment of AML is based on cytotoxic chemotherapy, differentiation therapy, and stem cell transplantation (SCT). The aim in chemotherapy is to suppress the leukemic cells to an inapparent level, in order to permit the normal polyclonal hematopoiesis. Differentiation therapy aims to promote cell maturation towards forms that lose their ability to proliferate in an uncontrolled fashion. In contrast, in SCT the sick bone marrow is replaced with a healthy one from the donor. All these treatments may eventually cure the patient.

1.2 Chemotherapy in the treatment of AML

AML is a marrow-based neoplasm where normal blood cells are replaced by malignant blasts. In most cases these blasts differentiate towards myeloid or monocytic lineage. Antileukemic therapy based on chemotherapy results in complete remission (CR) rates varying between 60 and 75%. It has proven difficult to improve the CR rates achieved with the different combinations of

cytarabine and anthracyclines. Long-term disease-free survival occurs in 25-50% of CR patients depending on post-remission therapy employed (Berman et al., 1991; Phillips et al., 1991; Schiffer et al., 1997; Zittoun et al., 1989).

1.3 Differentiation therapy

Another approach in achieving CR is differentiation therapy, where cells lose their ability to proliferate as they mature (Koeffler, 1983).
This approach has been proven to lead to remission in acute promyelocytic leukemia, where retinoic acid induce terminal differentiation of promyelocytic cells (Castaigne et al., 1990; Chen et al., 1991; Huang et al., 1988; Warrell et al., 1991). It has also been demonstrated that vitamin D analogs have an antileukemic effect in vitro and in vivo in mice (Honma et al., 1983; Zhou et al., 1990). Unfortunately, most of the vitamin D analogs raise the calcium level in blood, which has lead to hypercalcemia before antileukemic concentrations in blood have been reached (Koeffler et al., 1985).

1.4 Stem cell transplantation

The first successful bone marrow transplantations (BMT) were performed from human leukocyte antigen (HLA)-identical siblings in 1968 (Bach et al., 1968). Since then, what was once an experimental procedure has become established therapy. Traditional BMT from HLA-matched relatives has been supplemented with new methods of collecting hematopoietic stem cells from variety of sources and donors. Today, the stem cells can be harvested from bone marrow, peripheral blood, cord blood, and rarely fetal liver. The donor can be the patient him/herself (autologous transplant), an HLA-identical sibling or an HLA-matched volunteer (allogeneic transplant) perhaps living on a different continent.

Hematopoietic stem cell transplantation (HSCT) is increasingly used to treat both malignant and nonmalignant diseases (Goldman et al., 1998; Gratwohl et al., 2000; Tyndall and Gratwohl, 1997). Main indications in Europe 1998 were leukemias (33%), lymphomas (41%, mostly autologous), solid tumors (22%, 99% autologous) and non-malignant disorders (5%, 80% allogeneic). Absolute number of transplants per year has increased from 4234 in 1990 to 20892 in

1998 (Gratwohl et al., 2000). Due to lack of suitable related donors the proportion of unrelated, HLA-matched donors from international marrow-donor registries has increased.

1.5 Graft-versus-host disease (GVHD) and graft failure

The recognition of cells as self/foreign is based on histocompatibility antigens (Bach and Sachs, 1987). The HLA class I and II regions on chromosome 6 contain the most polymorphic genes in the human genome (Bodmer et al., 1999; Mach, 1994). The three class I (HLA-A, -B, -C) and eight class II (HLA-DRB1, -DRB3, -DRB4, DRB5, -DQA1, -DQB1, -DPA1, -DPB1) genes act as histocompability antigens causing transplantation barriers in stem cell transplantation as well as in solid organ transplantation. At least 456 different class I and 420 different class II alleles have been identified (Bodmer et al., 1999). This variability leads to the fact that it is extremely unlikely to find an HLA-identical, unrelated donor.

Antigens are recognized by T-cells only in conjunction with major histocompatibility complex (MHC), referred to as HLA in humans. In allogeneic SCT the immunocompetent cells within the graft react to host antigens, thus causing GVHD.

Increasing use of unrelated HLA-matched donors has leaded to higher incidence of GVHD (Ferrara and Deeg, 1991), that is a major cause of mortality and morbidity in these patients. Approximately 75% of transplant-related deaths are associated with GVHD (Brown et al., 1999) . Recently, a major goal has been to develop better HLA-typing techniques and new more effective prophylactic and therapeutic regimens that would overcome GVHD.

The most logical step to overcome GVHD is to identify and remove the main culprits, namely, T-cells. Therefore in the 1980s there was an attempt to deplete the T-cells from donor marrow to prevent GVHD. However, this has since been associated with a high risk of relapse (Goldman et al., 1988). The curative effect of allogeneic transplantation is, at least partly, mediated by graft-versus-leukemia effect. The hypothesis is further supported by the inverse correlation

between relapse and severity of GVHD (van Rhee et al., 1997), and by the observation that donor lymphocyte infusion can restore normal donor hematopoiesis in patients who have relapsed after allogeneic SCT (Apperley et al., 1998).

In conclusion, although T-cell depletion effectively prevents GVHD, it has an adverse effect on stem cell engraftment and increases the possibility of graft failures. GVHD is a major cause of mortality in allogeneic SCT, but some of it must be tolerated as it coexists with the vital graft-versus-leukemia effect.

1.6 Rejection of solid organ transplants

Transplantation of foreign tissue into a recipient leads to an antigen-specific immune response and non-specific inflammatory response. In graft rejection the main concern is the antigen-specific immune response, but this is amplified by the inflammatory response. The specific immune response is mediated by T cells and antibody-producing B cells. T cell activation is a key event in the immune response. Once activated by the antigen, T cells induce the differentiation and activation of various other cells.

T cells do not recognize free antigen, but short peptides presented by the antigen presenting cells (APC). These peptides become associated with MHC molecules of the APC prior to their transport to APC cell surface and the presentation to T cells. MHC class I molecules present peptides derived from endogenous proteins and are recognized by CD8 molecules on cytotoxic T cells. MHC class II molecules bind exogenous peptides and are recognized by CD4 molecules on helper T cells (Doyle and Strominger, 1987).

In direct presentation, recipient T cells recognize foreign peptides presented by donor APCs. These are mostly donor dendritic cells, bearing both MHC class I and II antigens, and rapidly migrating from the graft to recipient's lymphoid tissue. In indirect presentation, recipient T cells recognize foreign peptides processed and presented by recipient APCs.

T cell activation requires costimulatory signals. T cell receptor (TCR) linked with CD3 molecule binds to MHC-antigen complex expressed by the APC.

CD4/CD8 binds with MHC molecule and associates with TCR-CD3 complex. In addition, effective T cell activation requires costimulatory signals from CD28-B7 interaction and/or CD40-CD40 ligand interaction (Linsley et al., 1990; Sayegh and Turka, 1998). The activation of T cell is initiated when a required treshold number of triggered TCRs is reached. This treshold number varies depending whether costimulation exists (Viola and Lanzavecchia, 1996).

When CD28-B7 mediated costimulation is present, treshold number of triggered TCRs can be downregulated from 8000 to 1000 (Viola and Lanzavecchia, 1996). Cells able to provide CD28 costimulation are professional APCs such as macrophages and dendritic cells. These cells present the foreign antigen to T cells in lymphatic tissues. Recent research has focused on the mechanism of TCR activation in the constraints of APC and T cell membranes. The evidence indicates that many signaling components, such as Lck and LAT, are concentrated in cholesterol-enriched membrane microdomains, called rafts (Simons and Ikonen, 1997). Two studies demonstrated that rafts are required for efficient T cell activation and activated TCRs are recruited to the rafts (Montixi et al., 1998; Xavier et al., 1998).

B cells bind native antigen directly with their surface immunoglobulin receptors. The antigen is internalized, processed and presented with MHC class II molecule to CD4+ T cells. Activated T cells express CD40 ligand, which interacts with the CD40 molecule on the B cell, thus activating it (Linsley et al., 1990). The activated B cells proliferate and produce antibodies. Some B cells differentiate to plasma cells producing large amount of antibodies before their demise. Other B cells differentiate to memory B cells (Campbell and Halloran, 1996).

Rejection can be divided to hyperacute, acute and chronic rejection. Hyperacute rejection is mediated by preformed recipient antibodies to donor ABO blood group, MHC antigens or alpha-Gal epitope (Auchincloss and Sachs, 1998; Robson et al., 1999). Antibodies bind to graft causing fixation of complement, and attracting neutrophils (Hall and Suranyi, 1995; Hernandez-Fuentes et al., 1999). Complement forms a membrane attack complex (MAC), which causes an ion permeable hole to graft endothelial cell surface, thus disrupting the form and function of the cell membrane (Liszewski et al., 1996).

The result is rapid occlusion of the vasculature following widespread endothelial destruction, culminating in rapid graft failure. This is a rare phenomenon due to cross-matching tests done prior to the transplantation.

Acute graft rejection is mediated by T cells. Nude mice and T cell deprived animals fail to reject xeno- or allografts. Of the T cells, CD4+ cells have been found to be critical in initiating rejection. After the activation of the immune system many cells contribute to the acute rejection. Clinically the hallmark of acute rejection is a cellular infiltrate. This consists predominantly of small activated lymphocytes and monocytes/macrophages with some plasma cells, neutrophils and eosinophils. The infiltrate is histologically mostly perivascular with increasing manifestations in surrounding tissues as the rejection progresses (Hall and Suranyi, 1995). There is some question to the role of cytotoxic T cells and natural killer (NK) cells in acute graft rejection, although NK cells have clearly been shown to be involved in the rejection of bone marrow transplants (Yu et al., 1992).

Chronic rejection is characterized by vascular injury in the graft. Manifestations are fibrointimal arterial narrowing with surrounding tissue showing signs of ischemia. In cardiac transplants the process is atherosclerosis of coronary arteries. The lesions are less focal than in classical atherosclerosis (James et al., 2001; Paavonen et al., 1993). In renal grafts chronic rejection manifestates as interstitial fibrosis, tubular atrophy, glomerular sclerosis and basement membrane reduplication. De novo membranous glomerulopathy may also be a form of chronic rejection. All arteries in the transplanted kidney are affected by this process as can be demonstrated by angiography (Ponticelli, 2000). In liver grafts biliary ducts disappear in addition to arterial injury (Lowes et al., 1993). In lung grafts there is bronchiolar injury with bronchiolar occlusion (Boehler and Estenne, 2000; Date, 2001; Elssner and Vogelmeier, 2001).

In histology and immunohistochemistry T cells and macrophages can be identified, but not to the extent that can be seen in the infiltration of acute rejection. IgG and components of complement can also be found in the vessel walls. Common to all grafts is injury to vascular endothelium. Vascular endothelium of the graft is readily presented to host T cells and expresses the

ligands needed to activate and adhere them (class I and II MHC and ICAM-1). The endothelial cells can express surface molecules to facilitate the migration of lymphocytes and macrophages to surrounding tissues. Coagulation factors and growth factors such as platelet-derived growth factor (PDGF) and thrombin stimulate the growth of smooth muscle cells and fibroblasts. The "Response to injury" (Ross, 1993) hypothesis suggests that alloimmune-induced injury to the graft is low grade and repetitive, where repair and recovery is slower and can not keep pace with the injury. The manifestations of the injury are characteristic in each organ. These include obliterative bronchiolitis in lungs, glomerular injury in kidneys, and biliar injury in livers. The injury in chronic rejection may be difficult to differentiate from drug toxicity (e.g. cyclosporin in the kidney) or recurrence of the disease (e.g. atherosclerosis in the heart and primary biliary cirrhosis in the liver).

1.7 Limitations of immunosuppressive agents

Clinical immunosuppression has made giant steps of progress during the last two decades. The short term results are quite remarkable in preventing acute rejection and graft loss but the long term results have not been improved over the past two decades. Regardless of this development, chronic rejection (transplant arteriosclerosis) and chronic graft dysfunction remain a problem. Cyclosporin A combined with other immunosuppressive drugs forms the backbone of modern immunosuppressive treatment. Cyclosporin and tacrolimus may, albeit very effective in the prevention of acute rejection, cause graft dysfunction through scarring, particularly in kidneys (Mihatsch et al., 1998; Solez et al., 1998).

A new direction of low-toxicity immunosuppression has emerged. The goal is to minimize the severe side effects of drugs in use today: nephrotoxicity, neurotoxicity, development of cardiovascular diseases, infection, malignancy and adverse cosmetic changes.

Table 1. Immunosuppressive agents, their mechanism of action, and side effects

Cyclosporin A, CsA	Binds to cyclophilin. CsA cyclophilin complex blocks calcineurin-calmodulin-induced phosphorylation of the transcription factor for IL-2.	Nephrotoxicity, neurotoxicity, hypertension, lymphoproliferative disorders, other malignancies, post-transplant diabetes mellitus, viral infections, hirsutism and gingival hyperplasia.
Tacrolimus, FK506	Binds to FKBP-12. FK506-FKB-12 complex blocks calcineurin-calmodulin-induced phosphorylation of the transcription factor for IL-2. Mechanism is identical with CsA.	Nephrotoxicity, neurotoxicity, glucose homeostasis related problems, hypertension, lymphoproliferative disorders, other malignancies, post-transplant diabetes mellitus, viral infections, alopecia and pruritus. Insomnia, headache, tremor, muscle pain, fatigue and GI-symptoms are listed as less frequent side effects.
Mycophenolate mofetil, MMF	MMF is converted to MPA, a inhibitor of IMP-dehydrogenase, a key enzyme in "de novo" pathway of purine synthesis. The anti-proliferative action is mainly directed to lymphocytes, as they are highly dependent on this pathway.	Diarrhea, vomiting and opportunistic infections. Lymphoproliferative disease in approximately 1 %.
Rapamycin, Sirolimus, RPM	RPM binds to FKBP immunophilins, but has no affinity for the calcineurin-calmodulin complex. RPM blocks the 2nd set of phosphorylation in the late GI phase of the cell cycle.	Hypertriglyceridemia, hypercholesterolemia, leukopenia, thrombocytopenia, elevated liver enzymes and hypokalemia. Viral infections.
Steroids	1. anti-inflammatory: inhibition of phospholipase A2 and thereby arachidonic acid cascade (cyclo-oxygenase, thromboxane, prostacyclin) and 5-lipoxynase pathway. 2. inhibits APC function and transcription of several cytokines.	Poor wound healing , osteoporosis, avascular necrosis, cataracts, iatrogenic diabetes mellitus, obesity and hypertension. Cushingoid appearance and growth retardation in children.
Azathioprine	Anti-proliferative action. Blocks conversion of IMP to AMP and GMP, inhibition of PRPP amidinotransferase	Side effects from all rapidly dividing tissues, particularly bone marrow. Hepatotoxic. Potentially mutagenic.

2. The effect of vitamin D analogs on malignant cells

2.1 Induction of differentiation and inhibition of cell growth

AML cells are unable to differentiate to functional mature cells. The maturation of the cells is blocked at an early stage and the cells remain in a rapidly dividing proliferative pool. This arrest is caused by chromosomal alterations that affect transcription of genes involved in myeloid differentiation.

The maturation arrest of AML cells can be reversed in experimental models as demonstrated by Sachs and colleagues (Fibach et al., 1972; Sachs, 1978). In these experiments it was shown that myeloid leukemic cells can be differentiated to mature granulocytes and macrophages by macrophage/granulocyte inducer type 2, i.e. interleukin 6 (IL-6) (reviewed in (Sachs, 1990; Sachs, 1987)). Further success was obtained with IL-1, D-factor (also called HILDA and leukemia-inhibitory factor) (Sachs, 1995), retinoic acid (RA) (Breitman et al., 1980), tumor necrosis factor (TNF) (Peetre et al., 1986) and vitamin D_3 (Honma et al., 1983; Miyaura et al., 1981). The various results obtained in these experiments suggested that these differentiation inducers act by different mechanisms, and there are multiple differentiation pathways sharing common final stage/stages.

Most research thereafter has concentrated on all-trans retinoic acid and vitamin D_3. All-trans retinoic acid induces differentiation of several human leukemic cell lines and acute promyelocytic leukemia (APL) cells (Breitman et al., 1980; Castaigne et al., 1990; Chen et al., 1991; Huang et al., 1988). The physiologically active form of vitamin D_3 is $1\alpha25(OH)_2D_3$. It induces some myeloid leukemia lines to differentiate along the monocyte/macrophage pathway and prolongs the survival time of leukemic mice (Abe et al., 1981; Miyaura et al., 1981; Zhou et al., 1990). The biologic effects of RA and VD_3 are mediated by their nuclear receptors, retinoic acid receptor (RAR), retinoid X receptor (RXR), and vitamin D_3 receptor (VDR) (Evans, 1988). They are members of the steroid receptor superfamily and act as ligand-inducible transcription factors by binding to their cognate responsive DNA elements, known as RA-responsive element (RARE) and vitamin D_3-responsive element (VDRE) (Baker et al., 1988; Petkovich et al., 1987). RAR and VDR form heterodimers with RXR to bind

DNA with high affinity and effect transcriptional activation of target genes (Bugge et al., 1992; Kliewer et al., 1992; Yu et al., 1991).

Inhibition of the G1/S transition in the cell cycle induces differentiation and growth arrest of both HL-60 and U937 cells (Jiang et al., 1994; Schwaller et al., 1995). The $p21^{WAF1/CIP1}$ protein is a cyclin-dependent kinase (cdk) inhibitor that is one of the regulators of cell cycle progression in the G1/S transition. Incubation of HL-60 cells with $1\alpha25(OH)_2D_3$ causes transient overexpression of $p21^{WAF1/CIP1}$ (Schwaller et al., 1995). $p21^{WAF1/CIP1}$promoter contains a VDRE, and exposure of U937 myelomonoblasts to $1\alpha25(OH)_2D_3$ results in transcriptional activation of $p21^{WAF1/CIP1}$, probably through a ligand/VDR/VDRE interaction (Liu et al., 1996). There is also strong evidence that $p27^{KIP1}$ protein blocks entry to the S phase of cell cycle in $1\alpha25(OH)_2D_3$ treated HL-60 cells (Muto et al., 1999; Wang et al., 1996). Recently it was shown that a novel analog, Gemini-19-nor increased in HL-60 both the proportion of cells in the G(1)/G(0) phase and expression level of $p27^{kip1}$. Gemini-19-nor also stimulated expression of the potential tumor suppressor, PTEN. Furthermore, other inducers of differentiation, all-trans-retinoic acid and 12-O-tetradecanoylphorbol 13-acetate, increased PTEN expression in HL-60 cells. PTEN expression appeared to parallel terminal differentiation of myeloid cells (Hisatake et al., 2001). The end effect appears similar to results obtained with $p21^{WAF1/CIP1}$ above.

In addition to studies on leukemic cells there has been a great number of promising studies utilizing VD_3 or the novel analogs on other malignant cells (Shany et al., 2001). These include breast (Brenner et al., 1995; Chaudhry et al., 2001; James et al., 1994; James et al., 1998; Saunders et al., 1995; Yang et al., 2001), prostate (Peehl et al., 1994; Skowronski et al., 1995), colon (Diaz et al., 2000; Shabahang et al., 1994; Wali et al., 1995), squamous skin (Akutsu et al., 2001; Yu et al., 1995), and glioma cells (Naveilhan et al., 1994).

In summary, recent studies suggest that the ability of VD_3 and its novel analogs to induce differentiation and to inhibit growth of various malignant cells, is initiated through the activation or repression of target genes by VDR, and mediated through an increased expression of several cdk inhibitors.

2.2 Vitamin D analogs and apoptosis

Apoptosis is a Greek word meaning "to fall away from". It was chosen as it describes the microscopic membrane bound cell fragments, apoptotic bodies, resulting from programmed cell death. Apoptosis plays an important role in a variety of physiological processes. Defects in the regulation of apoptosis contribute to the course of many diseases. Cancer and autoimmune diseases where there is too little apoptosis and probably stroke and Alzheimer's disease when there is too much (Reed, 2000).

Apoptosis is caused by activation of a family of intracellular cysteine proteases which cleave their substrates at aspartic acid residues, known as caspases for cysteine aspartyl specific proteases (Alnemri et al., 1996). Many caspases are involved in apoptosis, a few are not. A subgroup of caspases: caspase 1, 4 and 5 process pro-inflammatory cytokines pro-IL-1β and pro-IL-18. The proteases match the cleavage sites within these pro-cytokines but not the cleavage sites of the proteins known to undergo cleavage during apoptosis (Reed, 1999; Salvesen and Dixit, 1997).

Two pathways for caspase activation have been described in detail: the death receptor or extrinsic pathway and the intrinsic pathway (Figure 1). In the extrinsic pathway, ligand binds to TNF-family receptor, such as TNFR1 and FAS. These receptors recruit adapter proteins to their cytosolic death domains (DD), which then bind death effector domain (DED)-containing pro-caspases, especially pro-caspase-8. This becomes caspase-8 which is shown to directly cleave and activate the effector protease, caspase-3 (Salvesen and Dixit, 1997; Wallach et al., 1999; Yuan, 1997). In contrast, intrinsic pathway involves mitochondria, which release cytochrome c into the cytosol triggered by various stimuli, including elevations in the levels of pro-apoptotic Bcl-2 family proteins such as Bax. In the cytosol cytochrome c binds and activates Apaf-1, which then binds and activates pro-caspase-9. Active caspase-9 activates caspase-3 similarly to caspase-8 in the extrinsic pathway (Green and Reed, 1998; Reed, 2000).

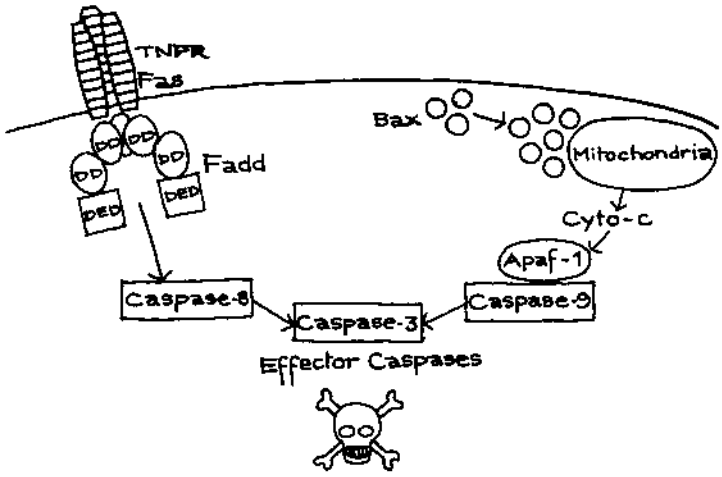


Figure 1. ***Extrinsic (left) and intrinsic (right) pathways for caspase activation.*** *The extrinsic pathway can be initiated by members of the TNF family of cytokine receptors, such as TNFR1 and Fas. These proteins recruit adapter proteins to their cytosolic death domains (DD), including Fadd, which then binds death effector domains (DED) containing pro-caspases, particularly pro-caspase-8. The intrinsic pathway can be initiated by release of cytochrome c from mitochondria. Induced by various stimuli, including elevations in the levels of pro-apoptotic Bcl-2 family proteins, such as Bax. In the cytosol, cytochrome c activates Apaf-1, allowing it to bind and activate pro-caspase-9. Active caspase-9 (intrinsic) and caspase-8 (extrinsic) have been shown to directly cleave and activate the effector protease, caspase-3.*

Numerous findings suggest the Bcl-2 family of proteins to be one of the key elements in deciding the fate of the cell. The anti-apoptotic Bcl-2 gene is activated by chromosomal translocations in the majority of non-Hodgkin's lymphomas (Tsujimoto et al., 1985) and is also overexpressed in many solid tumors (Reed et al., 1996). On the other hand, loss of function mutations have been identified in the pro-apoptotic Bax genes in many tumors. Analysis of Bax-knockout mice indicates that Bax is a tumor suppressor in vivo (Yin et al., 1997). These mice interestingly displayed a marked resistance to neuronal cell death induced by ischemia, axotomy and other insults. Bax is also increased in apoptosing neurons in Alzheimer's disease. Transcription of Bax is also indirectly regulated by p53, providing yet another connection in this complex pathway. In humans, 20 members of the Bcl-2 gene family have been described to date. These genes code the antiapoptotic proteins: Bcl-2, Bcl-X_L, Mcl-1, Bfl-1 (A1), Bcl-W and Boo (Diva) and pro-apoptotic proteins: Bax, Bak, Bok (Mtd),

Bad, Bid, Bim, Bik, Hrk, Bcl-X_S, APR (Noxa), p193, Bcl-G, Nip3, and Nix (BNIP) reviewed by Reed (2000). Some of these proteins are even products of the same gene through alternative mRNA splicing producing opposing end results on apoptosis (Bcl-X_L and Bcl-X_S).

Vitamin D_3 analogs induce apoptosis in many malignant cell types: leukemia (Elstner et al., 1996; Hisatake et al., 2001), myeloma (Park et al., 2000), breast cancer (Colston et al., 1992; James et al., 1998; Sundaram et al., 2000), prostatic cancer (Fife et al., 1997), colon cancer (Diaz et al., 2000; Evans et al., 1999), melanoma (Danielsson et al., 1998), glioma (Baudet et al., 1996), and cutaneus malignancies (Hershberger et al., 1999; Majewski et al., 2000). It remains still unknown how vitamin D_3 analogs induce apoptosis. Early reports stated that the VD_3 analogs induced apoptosis of myeloid leukemia cells while simultaneously down-regulating the expression of Bcl-2 protein suggesting an association between these two events. However, Grillier then reported that a VD_3 analog could induce apoptosis in a HL-60 variant without reduction in the cellular levels of Bcl-2 (Grillier et al., 1997).

On the other hand, a slight elevation in the levels of the pro-apoptotic Bax protein by vitamin D analogs has also been reported (Elstner et al., 1996). This slight elevation may be significant as apoptosis depends on the balance of antiapoptotic and pro-apoptotic factors. The former include the antiapoptotic members of the Bcl-2 family of proteins Bcl-2 and Bcl-X_L and the latter Bax and Bcl-X_S (Reed, 1998; Yang and Korsmeyer, 1996).

Interestingly, Mathiasen et al. reported that VD_3 analogs induced apoptosis via a novel caspase- and p53-independent pathway in MCF-7 and T47D human breast cancer cell lines. MCF-7 cells express a wild-type p53 tumor suppressor protein, while T47D cells lack a functional p53. In their experiments, VD_3 analogs induced growth arrest followed by apoptosis in both cell lines. Inhibition of caspase activation by several methods showed no effect on the induction of growth arrest or apoptosis by the VD_3 analogs under conditions in which apoptosis induced by TNF or staurosporine was effectively inhibited. Neither caspase-3-like protease activity nor cleavage of caspase substrate poly(ADP)ribose polymerase was detected in lysates from apoptotic cells following the successful VD_3 analog treatment. The VD_3-induced apoptosis

could be inhibited by a forced overexpression of Bcl-2 protein (Mathiasen et al., 1999).

Taken together, these findings suggest that the VD_3 analogs may use several pathways to induce apoptosis. However, the full understanding of the precise mechanism requires still further studies.

3. Vitamin D analogs in immunosuppression

3.1 Background

The role of $1\alpha25(OH)_2D_3$ in mineral and bone metabolism has been recognized and well characterized for a relatively long time (Norman et al., 1982). In the early 1980s several investigators described VD_3 receptors in many tissues not regarded to participate in mineral metabolism (Bhalla et al., 1983; Colston et al., 1981; Eisman et al., 1979; Feldman et al., 1980; Tanaka et al., 1982). This led to the conclusion that this hormone might have a broader function than previously appreciated.

Studies for immunoregulatory properties of vitamin D started. Tsoukas et al. demonstrated that $1\alpha25(OH)_2D_3$ suppressed the production of interleukin-2 (IL-2) and inhibited the proliferation of phytohemagglutin (PHA) stimulated lymphocytes (Tsoukas et al., 1984). Very soon thereafter Bhalla et al. reported similar results with antigen-induced T cell activation (Bhalla et al., 1984). Provvedini et al. found a nuclear VD_3 receptor in human B-lymphocytes. This was significant, as previous studies had described the receptor in the cytosol. In the same study they also demonstrated that VD_3 inhibited immunoglobulin (Ig) production of Epstein-Barr virus infected B-lymphocytes (Provvedini et al., 1986). A year later Reichel et al. demonstrated inhibition of γ-interferon synthesis by peripheral blood lymphocytes, using $1\alpha25(OH)_2D_3$ (Reichel et al., 1987). This inhibitive effect was shown to be independent of the levels of IL-2.

A real breakthrough in VD_3 immunosuppression came with the development of novel analogs. In 1991 Binderup et al. reported of the new 20-epi-vitamin D_3 analogs. These new molecules were exceptionally effective in inhibiting cell

proliferation and inducing differentiation but also in inhibiting T-cell activation *in vitro*. Many of these novel analogs were several orders of magnitude more active than CsA (Binderup et al., 1991). Literally hundreds of new analogs have been screened by various investigators globally, but only a few have shown real promise (Bertolini et al., 1999; van Etten et al., 2001).

3.2 Prevention of rejection

After encouraging results *in vitro* and in experimental autoimmune animal model, VD_3 analogs were further studied in solid organ transplantation. In 1991 Lemire et al. succeeded in prolonging the survival of murine cardiac allografts from 11 days to 27 days by the VD_3 analog $1,25(OH)_2$-Δ^{16}-cholecalciferol (Lemire et al., 1992).

In 1993 Veyron et al. prolonged skin allograft survival time in mice. The results were obtained with $1\alpha25(OH)_2D_3$, two different analogs CB966 and KH1060 as monotherapy or in combination with CsA. In the study, KH1060 was the most active compound, but the combination with CsA was able to prolong significantly graft survival more effectively than the use of either agent alone (Veyron et al., 1993).

A new 20-epi-vitamin D_3 analog, MC1288 was reported to be over 7000 times more potent in a mouse thymocyte costimulatory assay than $1\alpha25(OH)_2D_3$, and 300 times more potent in inhibiting allogeneic T-cell activation in a mixed lymphocyte culture (Binderup et al., 1991). Johnsson and Tufvesson studied this analog in heterotopic cardiac allograft model, heterotopic small bowel transplantation model, and in graft-versus-host reaction after small bowel transplantation. MC1288 significantly prolonged heart graft survival time in the study. It was also tested along with the immunostimulator LS-2616 (linomide), which totally abrogates the immunosuppressive effect of CsA. The survival time in the MC1288 treated group was halved by LS-2616, but clearly demonstrated that CsA and MC1288 exert their effects at least partly via a different mechanism. In small bowel transplantation the amount of hyaluronic acid secreted in the intestinal lumen was reduced thus indicating better graft function (Johnsson and Tufveson, 1994).

3.3 Prevention of GVHD

To our knowledge, there is only one study so far studying GVHD and the effects of vitamin D analogs. Johnsson and Tufveson studied the effects of MC1288 on graft-versus-host reaction after small bowel transplantation (Johnsson and Tufveson, 1994). In the untreated group signs of GVHD appeared around day 7, with progressive disease until death around day 14. Median in MC1288 group was 9,5 days (range 7-14). The two rats surviving for 14 days developed visible signs of GVHD later than the untreated, while the rest died without signs of GVHD. The authors concluded that the treatment had no beneficial effect on GVHD.

3.4 Prevention of autoimmune diseases

Effect of vitamin D analogs has been studied in several autoimmune disease models. Murine experimental autoimmune encephalomyelitis (EAE) is a primary T-cell mediated model of autoimmunity that is used as a model for multiple sclerosis. EAE can be easily induced in susceptible mice with central nervous tissue extracts. Within two weeks the animals develop acute paralysis with central nervous system inflammation (Paterson, 1976). Lemire and Archer managed to prevent the disease with the combination of $1\alpha25(OH)_2D_3$ and low calcium diet (Lemire and Archer, 1991). $1\alpha25(OH)_2D_3$ remarkably inhibited the induction of the disease, antibody production and development of histologic lesions.

Lemire et al. demonstrated the beneficial effect of VD_3 in the prevention of the histological signs of systemic lupus erythematosus (SLE) in an experimental murine lupus model. In this model MRL/l mice spontaneously develop lymphoid hyperplasia, dermatitis, vasculitis, arthritis, myocardial infarcts, and glomerulonephritis (Lemire et al., 1992).

Lillevang et al. experimented with the novel VD_3 analog KH1060 on mercuric-chloride-induced autoimmune rat model. This model is characterized by polyclonal activation of B-lymphocytes, proliferation of autoreactive CD4+ T-cells, and very high serum levels of IgE. Autoantibodies to the components of glomerular basement membrane such as laminin, type IV collagen and fibronectin lead to the development of glomerulonephritis resulting in severe

proteinuria, the most prominent feature of this model. While KH1060 as monotherapy was effective in this study, both KH1060 and CsA prevented the autoimmune reaction in a dose-dependent manner and their effects were found to be additive (Lillevang et al., 1992).

More recently Casteels et al. managed to prevent the recurrence of type 1 diabetes in syngeneic islet grafts in nonobese diabetic mice with the VD analog KH1060, both alone and in combination with CsA (Casteels et al., 1998). Van Etten et al. later demonstrated in a similar model that the combination of VD_3 analog TX527 with CyA and with IFNβ prolonged syngeneic graft survival significantly (van Etten et al., 2001).

AIMS OF THE STUDY

The aim of this study was:

1. To investigate the effects of vitamin D_3 analogs on the differentiation and growth of leukemia cells in vitro, and combinations of VD_3 analogs with chemotherapy in vitro.

2. To investigate VD_3 in the prevention of acute and chronic rejection in solid organ transplant model, and graft-versus-host disease in BMT model in vivo in rats.

METHODS

5.1 Materials

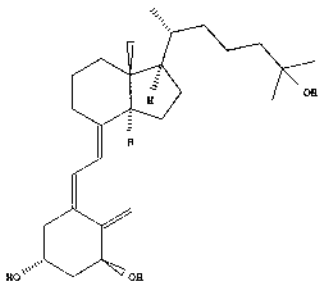
Cells (I, II)
The HL-60 cells are a cell line originally derived from a patient with acute myeloid leukemia (Collins, 1987; Collins et al., 1977). These cells have a capability to differentiate.

Bone marrow cells from AML patients were obtained from routine samples after informed consent according to institutional guidelines. Mononuclear cells were isolated by Ficoll-Hypaque density gradient and suspended in Iscove's modified Dulbecco's medium (IMDM; Sigma Diagnostics, St.Louis, MO, USA) with 10% fetal calf serum (FCS; Bioclear, Wilts, UK). Standard karyotype analysis on bone marrow cells was performed by G-banding technique.

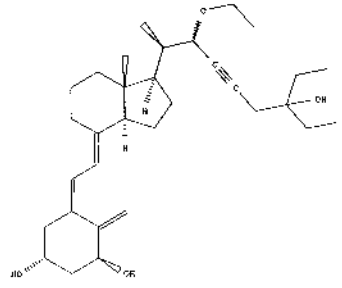
Vitamin D analogs (I, II, III, IV)

The following compounds were used in this study:

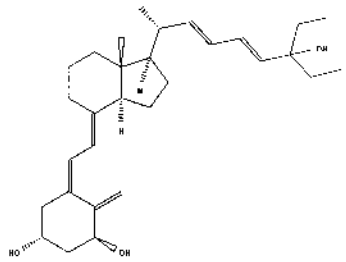
- 1(S), 3(R)-Dihydroxy-20(R)-(1(S)-1-ethoxy-5-ethyl-5-hydroxy-2-heptyn-1-yl)-9,10-seco-pregna-5 (Z), 7(E), 10(19)-triene (CB1093),
- 24a, 26a, 27a-tri-homo-22, 24-diene-1-alfa, 25-$(OH)_2$-D_3 (EB1089),
- 20-epi 1,25$(OH)_2D_3$ (MC1288), and
- 1α25$(OH)_2D_3$ (Figure 2).



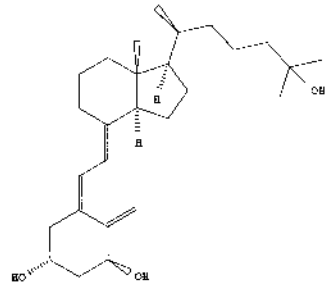
1,25 dihydroxyvitamin D3



CB1093



EB1089



MC1288

All compounds were synthesized at the Department of Chemical Research, Leo Pharmaceutical Products, Ballerup, Denmark. The compounds were supplied as solutions in isopropanol (4 x 10^{-3}M) and stored at -20^0C. For *in vitro* use, the analogs were diluted to IMDM (Sigma Diagnostics). For in vivo use, the analogs were diluted to phosphate buffered saline (PBS) with 0.02% rat albumin.

Experimental animals (III, IV)

Inbred rat strains DA, WF, BN and LEW were purchased from the Laboratory Animal Center, University of Helsinki, Helsinki. They received humane care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals prepared and formulated by the National Institute of Health (NIH Publication no. 86-23, revised 1985). Pellets (Altromin Nr. 1314, Standard diet, Chr. Petersen A/S, Ringsted, Denmark) were used as basic diet and tap water was given ad libitum.

5.2 In vitro experiments

Cell proliferation by methylcellulose colony forming assay (I, II)

$2x10^3$ HL-60 cells in exponential growth phase from liquid culture or $1x10^5$ AML cells were plated in 25 mm petri dishes (Nunc; Roskilde, Denmark) and cultured for 10 days. Cells were mixed in methyl cellulose (Fluka; Buchs, Switzerland), 1 ml containing 0.05 ml of 20%, BSA (Sigma), 1 µl of 2-mercapto-ethanol (Fluka), 0.2 ml of FCS (Bioclear), 0.165 ml of IMDM, and 0.1 ml of supernatant from human bladder cancer cell line 5637 grown as continuous culture in IMDM.

30 µl of different concentrations of vitamin D analogs were placed on the bottom of each dish. The total volume was 300 µl/well. Number of colonies (>40 cells/colony) was counted under inverted microscope. All experiments were done in triplicate with various concentrations of EB1089, CB1093 or idarubicin (IDA) (Pharmacia & Upjohn, North Peapack, NJ) and controls with no vitamin D analog or IDA added. Experiments with HL-60 cells were repeated at least three times.

Cell proliferation by ^{3}H-thymidine incorporation (I)

The cell solutions (grown in suspension culture flasks for a week with various concentrations of vitamin D analogs) were placed on 96 micro well tissue culture plates (Nunc), each well containing $2X10^5$ cells and 20 µl of 100 µCi ^{3}H-thymidine. Total volume in each well was 200 µl. The plates were harvested after 12-hour incubation at 37^0C using a Skatron Combi Cell Harvester. After

the samples were dried, 1 ml of OptiScint "HiSafe" (Fisons Chemicals, Loughborough, Leics, UK) was added to each test tube. Scintillation counts were measured with a beta counter (Rackbeta Liquid Scintillation Counter, LKB Wallac, Turku, Finland). Proliferation of cells was determined as percentages of controls.

Liquid culture and differentiation assays (I)

HL-60 cells differentiate towards macrophage-like cells when cultured in the presence of $1\alpha25(OH)_2D_3$. This differentiation can be measured by alpha-naphtyl acetate esterase (ANAE) activity in the cells and by their ability to reduce nitroblue tetrazolium (NBT). HL-60 cells were grown in suspension culture flasks (T-25, Falcon) containing IMDM and 10% FCS and different concentrations of different vitamin D analogs in humidified atmosphere with 5% CO_2 at 37^0C. Total volume in each flask was 5 ml. Cells were harvested after 7 days in culture. Induction of differentiation of HL-60 cells was measured both by reduction of NBT and by ANAE staining. For NBT, cell suspensions ($2x10^5$ cells/ml) were mixed with an equal volume of NBT solution containing 1,7 g of bovine serum albumin (BSA), 125 mg of NBT and 100 µg 12-0-tetradecanoylphorbol-13-acetatein in 100 ml of IMDM. The suspension was incubated for 30 min at 37^0C. Cells were washed twice in cold PBS, cytocentrifuged, fixed in methanol for 5 min and stained with Gram safranin for 10 min. Alpha-naphtyl acetate esterase kit (Sigma) was used for ANAE stainings, which were done according to the manufacturer's guidelines. All experiments were done at least three times with controls having no VD_3 analog added in cultures.

Mixed lymphocyte cultures (III)

To obtain rat lymphocytes WF rats were stimulated for 24 h by intravenous injection of 10×10^6 DA-strain spleen cells. Spleen cells were collected and then incubated overnight. Cells that were used for stimulation were irradiated. $2x10^6$ stimulator (DA) and 10^6 responder (WF) cells were incubated with various concentrations (10^{-6}M-10^{-11}M) of VD_3 analogs in 96 micro well tissue culture plates (Nunc). The following controls without vitamin D_3 analogues were used: irradiated stimulator cells, irradiated stimulator cells mixed with nonirradiated stimulator cells, and irradiated stimulator cells mixed with responder cells. After an incubation period of five days, 20 µl of 100 µCi ^{3}H-thymidine was

added to each well and the activity was measured with beta counter (Rackbeta Liquid Scintillation Counter). Proliferation of cells was determined as percentages of nontreated controls in four independent experimental sets.

Analysis of apoptosis (II)

$20x10^6$ HL-60 cells were incubated in suspension culture flasks (T-25, Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) containing IMDM, 10% FCS and different concentrations of EB1089 and CB1093 in humidified atmosphere with 5% $C0_2$ at 37^0C. Total volume in each flask was 5 ml.

After 72 hour incubation the contents of each flask were divided to five flasks, 4 ml of IMDM and IDA was added in concentrations from 10^{-9} M to 10^{-12} M and one flask with no IDA in each set of five. Resulting 25 flasks were incubated for 48 hours. Percentage of apoptotic, live and dead cells was evaluated using a commercial kit, Apoptosis Detection Kit (R & D Systems, Minneapolis, MN), according to manufacturer's instructions. Thereafter, the samples were immediately analyzed with flow cytometry (FACS IV, Becton Dickinson). The results are expressed as the percentage of live, necrotic and apoptotic cells, as counted with flow cytometry.

5.3 In vivo experiments

In vivo experiments on blood calcium levels (I)

Inbred DA (AG-B4, $RT1^a$) rats were divided in the following groups, each containing three rats: CB1093 0.1 ug/kg, CB1093 0.3 ug/kg, CB1093 1.0 ug/kg, $1\alpha25(OH)_2D_3$ 0.1 ug/kg, $1\alpha25(OH)_2D_3$ 0.3 ug/kg, $1\alpha25(OH)_2D_3$ 1.0 ug/kg. The treatment was administered intraperitoneally every other day. Serum calcium was measured by enzyme-linked immunosorbent assay (ELISA) from peripheral blood samples taken every seven days. The animals were sacrificed after five weeks.

Aortic transplantations (III)

Allogeneic transplants were performed from DA to WF strain. Rats weighing 250-350 grams and of 2 to 4 months of age were used as donors and recipients.

A segment of the descending thoracic aorta, approximately 3 cm in length, was excised, perfused with PBS and used as a transplant (Mennander et al., 1991). The rats were anesthetized with chloral hydrate, 240 mg/kg i.p., and 0.3 mg/kg s.c. buprenorphine (Temgesic, Reckitt & Colman, Hull, England) was used for post-operative pain relief. The graft was transplanted into a heterotopic position below renal arteries and above bifurcation in the abdominal cavity. The grafts were removed at 1 or 3 months posttransplantation, and processed for histology and frozen sections.

Vitamin D analogue, MC1288 (Leo Pharmaceutical Products) was given in three different doses: 0.1 µg/kg/every second day (esd) ip, 0.05 µg/kg/esd, and 0.01 µg/kg/esd. Cyclosporine A (CsA; Sandimmun Neoral, Sandoz, Basel, Switzerland) 5 mg/kg/d po, was used as a standard drug either alone or in combination with MC1288. The CsA levels were monitored weekly from peripheral blood samples using a radioimmunoassay kit for CsA (CYCLO-Trac, Incstar Corporation, Stillwater, Minnesota, USA).

Bone marrow transplantation (IV)

Allogeneic bone marrow transplantations were performed using adult Brown Norway (BN; $RT1^l$) rats as recipients and 10-week-old Lewis (LEW; $RT1^n$) rats as bone marrow cell donors. The cells were collected by flushing the femurs with saline. The recipient animals were irradiated with 960 rad (Varian Climac 600c, 6 MV photon irradiation, distance 120 cm, field size 40 x 40 cm^2) prior to BMT. After 24 hours, 60-80x10^6 mononuclear bone marrow cells from LEW rats were injected into BN recipients.

The animals were divided into four groups with the following treatments:

1) vitamin D analog MC1288 (Leo Pharmaceutical Products) 0.1 µg/kg/esd i.p.
2) CsA (Neoral; Novartis) 5 mg/kg/d, p.o.
3) MC1288 0.1 µg/kg/esd and CsA 5 mg/kg/d together
4) a control group receiving no immunosuppressive treatment.

CsA, which is used as a standard treatment in BMT patients, provided a secondary control group. Sulfonamide and trimethoprim (Cotrim; Merckle, Blaubeuren, Germany) was administered for ten days i.p. as infection prophylaxis to all recipients.

Histological analysis (III, IV)

(III) To study histological manifestations in the aortic allografts, the animals were sacrificed at 1 and 3 months post transplantation. Cross sections were stained with Mayer´s hematoxylin and eosin and prepared for quantitation of morphological changes in the graft. The changes were quantitated according to standard morphometric principles (Aherne and Dunnill, 1982) and expressed as point score units (psu), i.e., mean number of points falling over a given anatomical area using straight cross-sectional lines and a 0.02 mm grid. The number of nuclei in adventitia, media and intima and the thickness of intimal lesion, separated from each other by intimal and external elastic laminae were evaluated.

(IV) To study histological manifestations of GVHD, the animals were sacrificed 20 days after transplantation. After BMT from Lewis to BN rat strain, the strongest histological changes are observed in the liver, a model parenchymal target organ for acute GVHD in this strain combination (Renkonen and Häyry, 1984). Biopsies were taken from skin, liver and spleen, fixed in 4% formaline solution and processed for histology. Paraffin sections were stained with Mayer´s hematoxylin and eosin.

Histologic features of acute GVHD were graded independently by two investigators (I. Pakkala & E. Taskinen) on a 0 to 4 scale using previously published grading systems (Lerner et al., 1974; Renkonen and Häyry, 1984; Sale et al., 1977; Slavin and Santos, 1973). Briefly, skin manifestations were graded for their focal or diffuse vacuolar degeneration of epidermal basal cells (grade 1), diffuse spongiosis and dyskeratosis or eosinophilic degeneration of epidermal cells (grade 2). In grade 3 there were all above changes and partial separation of the dermal-epidermal junction, and in grade 4 loss of epidermis was seen. Liver manifestations were graded for small lymphocyte and plasma cell infiltration into the portal tracts, voluminous Kupffer cells, portal and/or central venous endothelialitis and evidence of bile duct injury or cholangitis. Spleen manifestations were graded for fibrosis and hypocellularity with an increase in siderophages and megakaryocytes. The results were expressed as the mean total increment (MTI) for each of the organs and summarized resulting in a maximum score of 12 reflecting the severity of acute GVHD.

Immunoperoxidase stainings (III)

For immunohistochemistry, 3-4 µm thick frozen sections were stained by an immunoperoxidase technique using monoclonal anti-rat antibodies to interleukin-2 receptor α-chain (CD25) (IL-2R; clone OX-39, PharMingen, San Diego, CA), to T helper cells (CD4; clone W3/25, Seralab, Sussex, UK), to cytotoxic T cells (CD8; clone OX8, Seralab), and to macrophages (ED1; Sero-Tec, UK). The cryosections were fixed with chloroform for 30 min, and then stained using standard 2-layer indirect immunoperoxidase technique. Intensity of the staining was evaluated using an arbitrary scale scored from 0 to 3 where 0 indicates no staining and 3 very strong staining.

(IV) Serial frozen sections were immunostained using a standard multi-layer method. Briefly, after acetone and chloroform fixation of the slides the nonspecific staining was blocked with appropriate 1.5% nonimmune serum (Vector Laboratories, Burlingame, CA). Primary antibodies are listed after their respective targets as follows CD4 (W3/25; Harlan Sera-Lab Ltd, Belton, England), CD8 (OX8; Sera-Lab), MHC class II common determinant (OX6; Sera-Lab), nitrate oxide synthase 2 (NOS2) (M-19; Santa Cruz Biotechnology, Santa Cruz, CA), and NKR-P1A (10/78; PharMingen, San Diego, CA). Secondary antibody was biotinylated and was followed by avidin-biotin complex (Vectastain Elite ABC Kit, Vector Laboratories) and chromogen 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, USA) containing 0.4% hydrogen peroxidase, yielding a brown-red reaction product. The specimens were counterstained with hematoxylin.

Immunostaining was evaluated by two observers and results were expressed as mean number of positive cells/visual field at magnification 1000x.

In vivo proliferation assay by bromodeoxyuridine (BrdU)

(III) The rats received 300 µl of bromodeoxyuridine (BrdU, Zymed Laboratories) by i.v. injection 4 hours before sacrifice. Frozen sections of aortic allografts were used for immunoperoxidase staining by BrdU primary antibody. First, the sections were digested with 0.1% pepsin in 0.1 M HCl for 30 min. Thereafter they were fixed in 95% volume percent of formamide in

0.15 M tri-sodium-citrate in +70°C for 45 min, and washed three times with PBS. Blocking of unspecific staining was performed using horse serum for 30 min. The primary antibody for BrdU (Mouse anti-bromodeoxyuridine, Bu20a, Dako Immunoglobulins A/S, Glostrup, Denmark) was used at a dilution of 1:20; thereafter Vectastain ABC-kit (Vectastain ABC-kit Elite PK-6102, mouse IgG, Vector Laboratories Inc., Burlingram, CA) was used according to manufacturer's instructions. The frozen sections were counterstained and scored as labeled cells/cross section.

5.4 Statistical methods

(I, II, IV) Statistical analysis was performed using the non-parametric Kruskall-Wallis test with Dunn correction for multiple comparisons, where $p<0.05$ was considered significant.

(III) The nonparametric Mann-Whitney U test was used in comparison of two groups and Kruskall-Wallis test with Dunn correction for multiple nonparametric groups.

RESULTS

6.1 CB1093, a novel vitamin D analog; effects on differentiation and clonal growth on HL-60 and de novo leukemia cells (I)

The effects of vitamin D analogs on differentiation of HL-60 cells

All three analogs demonstrated a dose dependent effect on HL-60 cells. ANAE stainings showed CB1093 to be 3.5 times more potent than EB1089 and 45 times more potent than $1\alpha25(OH)_2D_3$ in the induction of esterase (ED_{50} $2x10^{-9}$, $7x10^{-9}$, and $9x10^{-8}$, respectively). The reduction of nitroblue tetrazolium (NBT) correlates with the induction of superoxide production of differentiated HL-60 cells. Here we showed that CB1093 reduced NBT 50 times more efficiently than EB1089 and 30 times more efficiently than $1\alpha25(OH)_2D_3$ (ED_{50} $1x10^{-9}$, $5x10^{-8}$, and $3x10^{-8}$, respectively).

The effect of vitamin D analogs on proliferation of HL-60 cells

In methyl cellulose colony assays CB1093 was 7 times more efficient than EB1089 and 10 times more efficient than $1\alpha25(OH)_2D_3$ in inhibiting proliferation (ED_{50} $9x10^{-11}$, $6x10^{-10}$, $9x10^{-10}$, respectively). The proliferation of HL-60 cells measured by 3H-thymidine incorporation showed CB1093 to be 3 times more potent in inhibiting cell proliferation than EB1089, and 20 times more potent than $1\alpha25(OH)_2D_3$ (ED_{50} $3x10^{-9}$, $9x10^{-9}$ and $6x10^{-8}$, respectively).

Table 2. Relative efficacies of selected vitamin D analogs as compared to $1\alpha25(OH)_2D_3$

	Relative efficacy		
Analog	ANAE	NBT	Inhibition of clonal growth
$1\alpha25(OH)_2D_3$	1	1	1
CB 1093	450	300	100
EB1089	12.9	0.6	1.5
*1,25$(OH)_2$-16ene-23yne-D_3	120	20	1.8

Relative efficacy is calculated by:

$$\frac{\text{concentration of analog to elevate serum } Ca^{++}}{\text{concentration of } 1\alpha25(OH)_2D_3 \text{ to elevate serum } Ca^{++}} \times \frac{\text{ED50 of } 1\alpha25(OH)_2D_3 \text{ in ANAE, NBT or colony assay experiments}}{\text{ED50 of analog in ANAE, NBT or colony assay experiments}}$$

Abbreviations: NBT, nitroblue tetrazolium; ANAE, alpha-naphtyl acetate esterase; ED50 represents the effective dose achieving 50% response.
*As reported in (Zhou et al., 1989)

The effect of vitamin D analogs on de novo leukemia cells

CB1093 was even more effective against de novo AML cells under similar conditions. In methyl cellulose colony assays CB1093 was 25 times more efficient than EB1089 and about 100 times more efficient than $1\alpha25(OH)_2D_3$ in inhibiting the proliferation of AML cells (ED_{50} $5x10^{-11}$, $8x10^{-10}$ and $6x10^{-9}$, respectively).

Effect on blood calcium

$1\alpha25(OH)_2D_3$ and EB 1089 have been shown to have similar calcemic effects (Pakkala et al., 1995). CB1093 had a much lesser effect on calcium metabolism than $1\alpha25(OH)_2D_3$ when given intraperitoneally. The changes in S-Ca values for the groups receiving high, medium and low i.p. doses (1.0 ug/kg, 0.3 ug/kg and 0.1 ug/kg) of CB1093 were 2.50-2.63 mmol/l, 2.48-2.50 mmol/l and 2.52-2.33 mmol/l, respectively. Thus, CB1093 did not cause hypercalcemia with the doses used in this study.

The groups receiving high and medium doses (1.0 ug/kg and 0.3 ug/kg) of $1\alpha25(OH)_2D_3$ developed hypercalcemia during the experiment (S-Ca: 2.51-2.92 mmol/l and 2.50-2.71 mmol/l, respectively). In the group receiving 0.1 ug/kg

of $1\alpha25(OH)_2D_3$, S-Ca rose only from 2.50 to 2.62 mmol/l. Our preliminary results with short oral administration suggest that CB1093 has 25% of the calcemic effect of $1\alpha25(OH)_2D_3$.

6.2 Vitamin D analogs EB1089 and CB1093 combined with idarubicin inhibited proliferation and promoted apoptosis in HL-60 leukemia cells (II)

Combined use of vitamin D analogs and IDA effectively inhibits HL-60 cell proliferation

To study whether combining novel vitamin D analogs with IDA would inhibit HL-60 cell proliferation more effectively than IDA alone, methylcellulose colony forming assays were performed in various concentrations of these drugs (Table 3). Untreated control HL-60 cells formed 301±12 colonies on petri dishes. IDA alone at concentration 10^{-12} M permitted growth of 270±8 colonies whereas at concentration 10^{-10} M or higher, all colony forming was prevented. Combining vitamin D analog, EB1089, at concentration 10^{-10} M with IDA decreased the number of colonies to 200±14 ($p<0.0001$) whereas higher concentration at 10^{-8} M further inhibited the growth to 61±4 colonies ($p<0.0001$). Similar results were observed with another vitamin D analog, CB1093, where 169±9 colonies (10^{-10} M), and 73±3 colonies (10^{-8} M) were formed ($p<0.0001$). However, the inhibition of cell proliferation was more pronounced in any of the treatment groups than in untreated controls ($p<0.0001$). In addition, when comparing efficacy of these two vitamin D analogs without IDA, CB1093 showed to be more effective than EB1089 in preventing colony forming at concentration 10^{-8} M ($p<0.0001$).

Table 3

Cell proliferation, as measured with methyl cellulose colony forming assays. The results are expressed as number of cell colonies (>40 cells), counted under inverted microscope. Mean values of three experiments ± SD, each done in triplicate.

	EB1089 10^{-8} M	**EB1089 10^{-10} M**	**CB1093 10^{-8} M**	**CB1093 10^{-10}M**	**No vitamin D analog**
No Ida	110 ± 9	243 ± 6	90 ± 4	228 ± 7	301 ± 12
Ida 10^{-12} M	61 ± 4	200 ± 14	73 ± 3	169 ± 9	270 ± 8
Ida 10^{-11} M	9 ± 3	21 ± 1	5 ± 2	14 ± 3	50 ± 3
Ida 10^{-10} M	0	0	0	0	0
Ida 10^{-9} M	0	0	0	0	0

In conclusion, combining vitamin D analogs, EB1089 or CB1093 with IDA inhibited more effectively HL-60 cell proliferation and colony forming than using IDA alone.

Vitamin D analogs induce apoptosis rather than necrosis in HL-60 cells

This experiment aimed to study the effects of sequential treatment with a vitamin D analog and IDA. The cells were first incubated with the analog for 72 h. IDA was then added and incubation continued for 48 h. The results are expressed as the percentage of live, dead and apoptotic cells, as counted with flow cytometry (Table 4).

Preincubation of HL-60 cells with vitamin D analogs, EB1089 or CB1093, induced programmed cell-death, i.e., apoptosis in 80% and 72% of the cells, respectively, compared to 30% in untreated controls ($p<0.0001$). At the same time, the pool of dead cells was decreased from 8% to 4% or 3%, respectively ($p<0.001$). Vitamin D analogs were tested in two different concentrations, 10^{-8} and 10^{-10} M. EB1089 significantly increased the number of apoptotic cells in both concentrations compared to untreated controls. In contrast, CB1093 was effective only in the higher concentration.

Table 4

The results are expressed as the percentage of cells ± SD, as counted with flow cytometry.

Apoptotic	EB1089 10^{-8} M	EB1089 10^{-10} M	CB1093 10^{-8} M	CB1093 10^{-10}M	No vitamin D analog
No Ida	80.2 ± 4.5	44.4 ± 1.1	72.3 ± 0.7	26.8 ± 0.4	29.8 ± 0.6
Ida 10^{-12} M	73.1 ± 1.1	57.0 ± 1.3	73.0 ± 0.8	38.5 ± 0.8	32.6 ± 0.3
Ida 10^{-11} M	80.0 ± 1.8	47.6 ± 0.8	77.0 ± 0.5	31.0 ± 4.9	27.9 ± 0.4
Ida 10^{-10} M	81.4 ± 0.6	71.3 ± 1.1	79.6 ± 0.4	67.9 ± 3.6	67.3 ± 0.7
Ida 10^{-9} M	7.1 ± 0.1	2.0 ± 0.1	3.7 ± 0.2	2.8 ± 0.2	1.7 ± 0.3

Necrotic	EB1089 10^{-8} M	EB1089 10^{-10} M	CB1093 10^{-8} M	CB1093 10^{-10}M	No vitamin D analog
No Ida	4.3 ± 0.5	4.5 ± 0.5	3.0 ± 0.2	2.7 ± 0.4	7.9 ± 0.2
Ida 10^{-12} M	2.9 ± 0.1	6.7 ± 0.2	3.9 ± 0.3	2.0 ± 0.2	5.4 ± 0.2
Ida 10^{-11} M	3.6 ± 0.6	7.9 ± 0.7	2.2 ± 0.1	4.6 ± 0.7	5.9 ± 0.4
Ida 10^{-10} M	11.0 ± 0.2	13.7 ± 0.5	8.0 ± 0.4	8.2 ± 0.5	20.9 ± 0.4
Ida 10^{-9} M	91.9 ± 0.3	90.6 ± 0.4	93.4 ± 0.5	93.5 ± 0.6	90.3 ± 0.2

Live	EB1089 10^{-8} M	EB1089 10^{-10} M	CB1093 10^{-8} M	CB1093 10^{-10}M	No vitamin D analog
No Ida	15.2 ± 2.6	44.4 ± 0.8	24.4 ± 0.5	67.9 ± 0.7	60.7 ± 0.4
Ida 10^{-12} M	23.6 ± 0.8	35.8 ± 2.2	22.9 ± 0.4	57.8 ± 0.4	60.3 ± 0.4
Ida 10^{-11} M	16.0 ± 0.8	43.3 ± 0.8	20.6 ± 0.4	61.5 ± 7.4	62.0 ± 0.4
Ida 10^{-10} M	7.2 ± 0.2	14.6 ± 1.3	12.2 ± 0.3	23.6 ± 3.4	11.5 ± 0.3
Ida 10^{-9} M	0.9 ± 0.1	0.5 ± 0.1	2.2 ± 0.3	0.8 ± 0.2	0.4 ± 0.1

When using IDA alone at concentrations 10^{-12} M and 10^{-11} M it appeared to be ineffective, at concentration 10^{-10} M the increase in the pool of apoptotic cells was observed (30% vs. 67%). This was, however, associated with a rapid increase in the number of dead cells as well (8% vs. 21%). At concentration above 10^{-10} M IDA appeared to be cytotoxic killing 90% of the cells. Taken together, the optimal concentration for IDA showed to be 10^{-10} M in this experiment.

When pretreating the HL-60 cells with EB1089 10^{-8} M or CB1093 10^{-8} M the number of apoptotic cells increased compared to IDA alone (81% or 80% vs. 67%, $p<0.0001$). This effect was not , however, significantly better than using EB1089 10^{-8} M alone (80%, $p=0.3825$). More importantly, the cell death induced by IDA was significantly decreased by pretreatment (21% vs. 11% or 8% dead cells, respectively, $p<0.0001$). Number of live cells was similar in all groups.

These experiments have demonstrated that novel vitamin D analogs, EB1089 and CB1093 were effective in inducing programmed cell-death in HL-60 cells. Pretreating the cells with analogs prior to the exposure to IDA decreased the number of necrotic dead cells, while simultaneously increasing apoptosis.

6.3 MC1288, inhibits adventitial inflammation and suppresses intimal lesions in rat aortic allografts (III)

Effect of MC1288 on rat mixed lymphocyte culture (MLC)
We initially tested the effect of MC1288 on lymphocyte proliferation in vitro using MLC. Rat MLC demonstrated that MC1288 was 100-fold more effective than native vitamin D in suppressing stimulated lymphocytes in vitro.

Effect of MC1288 and CsA on allograft arteriosclerosis
To evaluate the effect of MC1288 on preventing acute and chronic rejection in vivo we performed rat aortic allografts. The rats were treated either with MC1288 or CsA alone or in combination. Even as late as three months after transplantation, there was still some adventitial inflammation. Disappearance of media cells (smooth muscle cells) and development of intimal thickening with an increase in cellularity in the neointima was observed in nontreated allografts. The MC1288+CsA treatment suppressed these manifestations of allograft arteriosclerosis, although it did not abolish them completely.

Administration of MC1288 or CsA alone slightly decreased the number of inflammatory cells in the adventitial layer of the graft. However, the most

effective inhibition was obtained when MC1288 and CsA were combined. The number of cells in the adventitia decreased from 10.0±0.9 to 4.1±1.0 psu, $p<0.01$. Another manifestation of allograft arteriosclerosis, disappearance of SMC in media, was almost completely abolished by all treatment groups. In addition, the most important feature of allograft arteriosclerosis clinically, intimal thickening, was decreased in all treatment groups. MC1288 or CsA alone decreased intimal thickening from 2.5±0.3 to 0.8±0.2 psu respectively, $p<0.01$, and combined treatment to 1.1±0.4 psu.

Proliferation assay of adventitial cells in vivo

To evaluate the effect of MC1288 on lymphocyte proliferation in vivo we administrated BrdU and detected with immunoperoxidase staining the number of positive cells in cross section. MC1288+CsA treatment did not only decreased the number of adventitial cells but also their replication activity from 140±36 to 20±19 labeled cells/cross section at one month after transplantation. However, there was no longer any difference in proliferation rate at three months.

Inflammatory pattern of adventitial cells modified by MC1288

The pattern of adventitial inflammatory cells was investigated at one month after transplantation. Treatment with MC1288 decreased the number of cells expressing interleukin 2 receptor (IL-2R) from 156±32 to 90±21 positive cells/cross section in the vascular wall, as well as the intensity of the staining of T helper cells (CD4). The expression of cytotoxic T cells (CD8) was not affected but the expression of macrophage marker ED1 was slightly upregulated.

Side-effects

MC1288 did not induce hypercalcemia during the first month of treatment, but in the long term experiment (3 months), the serum calcium level increased from 2.4±0.1 mmol/l (age-matched nontreated controls) to 3.2±0.2 mmol/l, $p<0.05$, in the MC1288 group, and to 3.3±0.3 mmol/l, $p<0.05$, after combined treatment with MC1288+CsA. Thus adding CsA into the protocol did not affect the induction of hypercalcemia.

Another side-effect observed was that the animals treated with MC1288 or MC1288+CsA lost weight during three weeks from 197±27 to 160±26 grams (18.5%) and from 155±16 to 147±18 grams (4.7%), respectively. During the observation time untreated control animals remained the same weight (198±18 grams). One of MC1288 treated animals was lost before the end of follow-up time due to hypercalcemia and cachexia.

6.4 MC1288, a vitamin D analog, prevents acute graft-versus-host disease in rat bone marrow transplantation (IV)

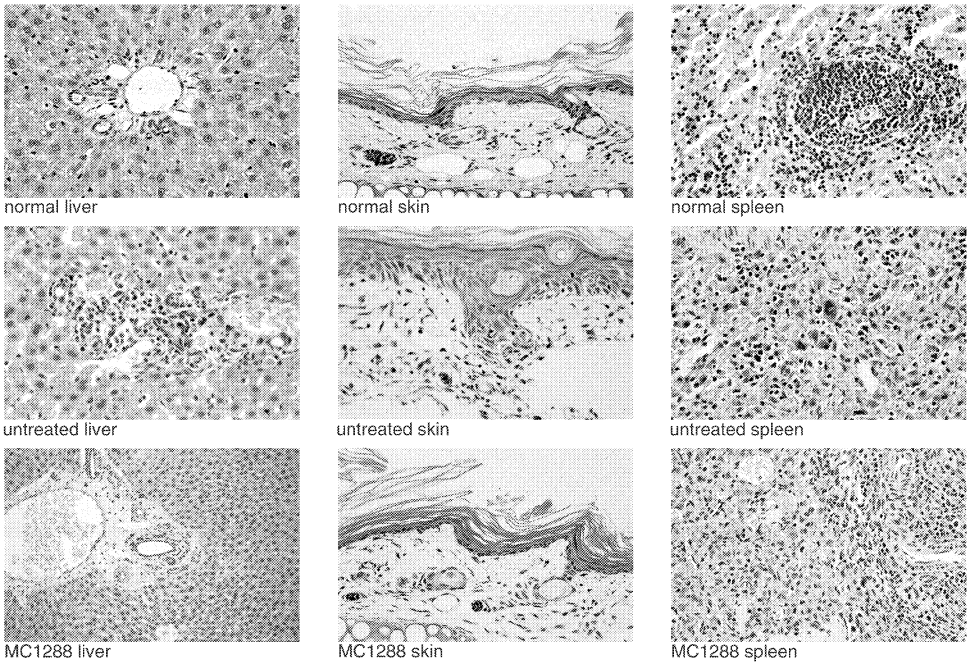
MC1288 effectively suppressed GVHD related dermatitis

Acute GVHD manifests first and most commonly in the skin. After allogeneic BMT, the untreated control group showed signs of dermatitis in the paws and the erythema of the ears from day five onwards. The animals began to lose hair on day 11. The CsA treated group showed similar, although milder, signs of GVHD within the same time frame. Rats that received MC1288 or the combined treatment with CsA showed none of the above signs of GVHD.

All animals developed mild diarrhea by day 9. In addition, they lost some weight. Before BMT, the recipient rat weights were 255± 40g, but decreased to 191±14 g in the untreated, to 219±25 g in the CsA, to 228±39 g in the MC1288 and to 225±27 g in the combined treatment group. Taken together, MC1288 effectively suppressed the clinical signs of GVHD, i.e., dermatitis, weight loss and hair loss in rats after BMT.

MC1288 suppressed histological manifestations of GVHD

Within 20 days after BMT, the untreated rats developed classical signs of GVHD in various organs (Figure 3). In the liver, the portal tracts were enlarged and affected by moderate inflammatory infiltration and edema, and there was mild cholangitis. In contrast, in the MC1288 treated animals the portal tracts were only slightly enlarged with little to no inflammation or destruction, but with mild inactive fibrosis in the background.



In the untreated rats, the ear skin manifestations of GVHD included markedly affected epidermis with intercellular edema, destructed basal cells and some intraepithelial lymphocytes. In contrast, in the ear skin of the MC1288 treated animals, there was no inflammation, edema or destruction. However, some mild degeneration of the basal layer was observed.

In the spleen of the untreated rats, a complete loss of normal splenic architecture of white and red pulp was seen. There were pronounced reactive alterations including fibrosis, depletion of lymphocytes in the white pulp and the appearance of megakaryocytes and siderophages in the red pulp. In contrast to manifestations in the liver and skin, the MC1288 treated animals showed similar, but less pronounced, alterations in the red and white pulp. Several siderophages but no megakaryocytes were found in the red pulp.

In the group that received MC1288 and CsA combined, the histological signs of GVHD were essentially similar to that of MC 1288 alone (data not shown). In contrast, CsA alone at the dose of 5 mg/kg/d had little effect on preventing the GVHD lesions (data not shown).

To assess the degree of GVHD, we graded these histologic alterations of GVHD in the skin, liver , and spleen in different treatment groups as a mean total increment. The groups treated with MC1288, either alone, or in combination with CsA, had a significantly lower mean total increment (1.4±0.5 and 1.1±0.6, respectively) compared to the untreated animals (5.0±1.6, $p<0.001$ for both). CsA alone was slightly beneficial (3.5±1.0, p=ns), albeit not significantly, compared to the untreated rats.

Taken together, these results show that a novel vitamin D analog, MC1288, was effective in preventing histological manifestations of acute GVHD in the skin, liver , and spleen. MC1288 was more effective than CsA alone in preventing GVHD, but when combined these two drugs may have even additive effects.

MC1288 suppressed immune cells more effectively than CsA in BMT

To study the effect of MC1288 on effector T-cells and macrophage activation, immunohistochemistry was performed on frozen sections of liver and skin and quantified. The results are summarized in Table 5.

In the liver, MC1288 markedly suppressed the expression of CD4 positive cells. The effect was more pronounced when combined with CsA. MC1288 and CsA were equally effective in suppressing CD8 positive cells. However, when the animals were treated with the combination of these drugs, no CD8 positive cells were found. The findings were similar in the MHC class II expressing cells. IL-2 receptor (IL-2R) expression was strongly suppressed after MC1288 treatment as well as after CsA and CsA+MC1288 treatments. Nitric oxide synthase 2 (NOS2) in the liver was suppressed in all treatment groups. The expression of natural killer cells (NKR-P1A) was markedly suppressed with the combination therapy, whereas MC1288 alone had a lesser effect. The number of NK cells in the CsA group was slightly elevated compared to the untreated group. To summarize, MC 1288 effectively suppressed CD4, CD8, MHC class II, IL-2R , and NOS2 expression in the liver.

Table 5. Expression of inflammatory markers in liver and skin after BMT. Data is expressed as the number of positive cells/high power field ±SD where *p<0.001 comparing to untreated group.

	MC1288	MC1288+CsA	untreated	CsA
Liver				
CD4	2.2±0.3*	0.8±0.2*	6.4±0.6	3.4±0.2*
CD8	0.8±0.2*	0.0±0.0*	3.6±0.3	0.8±0.1*
MHC class II	11.7±0.4*	7.3±0.2*	23.2±0.9	13.6±1.2*
IL-2R	0.0±0.0*	0.1±0.0*	1.3±0.1	0.0±0.0*
NOS2	0.1±0.1*	0.0±0.0*	1.2±0.2	0.0±0.0*
NKR-P1A	0.3±0.0	0.0±0.0*	0.4±0.0	0.6±0.1*
Skin				
CD4	0.4±0.1*	0.2±0.1*	2.8±0.4	0.6±0.1*
CD8	0.0±0.0*	0.0±0.0*	1.3±0.2	1.2±0.2
MHC class II	3.6±0.5*	1.6±0.1*	13.4±3.0	4.7±0.3*
IL-2R	0.1±0.1*	0.0±0.0*	2.2±0.2	0.9±0.3*
NOS2	0.1±0.1*	0.7±0.1*	1.1±0.1	1.7±0.1*
NKR-P1A	0.0±0.0*	0.0±0.0*	1.9±0.2	0.2±0.0*

In the skin, MC1288 markedly suppressed the expression of CD4 positive cells but the effect was more pronounced when combined with CsA. CD8 positive cells were totally suppressed by MC1288. CsA did not have a significant effect on CD8 cells in the skin. The MHC II expression in the skin was very similar to the liver. MC1288 proved to be very effective in suppressing class II expression with a further improved effect in combination therapy. IL-2R expression was also significantly suppressed in the skin after MC1288 treatment. NOS2 expression in the skin was markedly decreased by MC1288. Interestingly, in the skin, CsA treatment caused an unexpected elevation of NOS2 expression as opposed to the effect in the liver. This finding was concordant with the fact that combination treatment showed more positivity than MC1288 alone. NK cells were absent in both of the MC1288 treated groups and the CsA alone group was also significantly lower. In summary, MC1288 effectively suppressed CD4, CD8, MHC class II, IL-2R, NKR-P1A , and NOS2 expression in the skin 20 days after allogeneic BMT.

DISCUSSION

7.1 Low calcemic activity and good antileukemic potential make CB1093 a promising candidate for phase I clinical studies

We had previously shown that EB1089 is one of the most potent inducers of differentiation of HL-60 cells (Pakkala et al., 1995). The novel analog CB1093 proved to be an even more potent differentiating agent than EB1089. The comparison of the efficacy of the analogs on the colony forming potential and DNA synthesis of HL-60 cells showed CB1093 also to have the highest capacity to inhibit leukemic cell growth. The ED50 values for $1\alpha25(OH)_2D_3$ and EB1089 were comparable with results reported previously. Thus, it was possible to make comparisons also with analogs reported elsewhere (Zhou et al., 1989).

So far only two analogs (EB1089 and $1,25(OH)_2$-16ene-23yne-D_3) tested both in vitro and in vivo had shown promise for clinical trials. It is important to notice that for therapeutic purposes a vitamin D analog has to show not only good differentiating capacity and antileukemic effect but also low calcemic effect.

CB1093 was at least equally effective as the best analogs reported so far in inducing differentiation of HL-60 cells. It was even more effective against de novo AML cells. The consistent effect against AML cells suggested that CB1093 might have antileukemic potency also in vivo. CB1093 is better tolerated than most vitamin D analogs. The best antileukemic analog so far has been EB1089 but our study suggested that CB1093 could be given in doses ten times higher than EB1089. This dose would result in antileukemic efficacy 100 times higher than achieved with $1\alpha25(OH)_2D_3$, assuming that the pharmacokinetics are comparable. Comparison to other analogs tested in our study or reported previously also demonstrated the superiority of CB1093.

7.2 The combination of EB1089 or CB1093 with idarubicin

CR rates achieved with different combinations of cytarabine and anthracyclines have been unimproved over the past decade. A novel approach to improve the CR rates would be the combination of existing anthracycline therapy with differentiation therapy as used in APL.
APL differs from all other AML subtypes due to its unique 15;17 translocation. Differentiation therapy is targeted to the disrupted gene function of this translocation. Noncalcemic vitamin D analogs EB1089 and CB1093 have antileukemic effects in vitro on different AML subtypes and could extend the differentiation therapy to other AML subtypes as well. We demonstrated in this experiment that combination treatment of EB1089 or CB1093 with IDA inhibited more effectively the proliferation and colony forming of HL-60 cells than IDA alone. This would allow the reduction of anthracycline doses, and thereby reduce the adverse cardiotoxic effects of these drugs without jeopardizing the remission rates.

Alternatively, one could use vitamin D analog sequentially with anthracyclines to utilize the differentiation promoting properties of vitamin D analogs. Some recent studies have reported that induction of differentiation is associated with increased resistance to apoptosis-inducing agents, such as chemotherapy and γ-irradiation (Ketley et al., 1997). Our studies supported this notion. We demonstrated that preincubation of HL-60 cells with vitamin D analogs EB1089 or CB1093 prior to exposure to IDA induced significantly more programmed cell-death, i.e., apoptosis in these cells. However, compared to the effect of vitamin D analogs alone the effect on apoptosis was not enhanced. At the same time the number of necrotic cells was slightly diminished.

These experiments have demonstrated that novel vitamin D analogs, EB1089 and CB1093 were effective in inducing programmed cell death in HL-60 cells. Pretreating the HL-60 cells with vitamin D analogs prior to the exposure to IDA decreased the number of necrotic cells, while simultaneously increasing apoptosis. Combining vitamin D analogs, EB1089 or CB1093 with IDA inhibited HL-60 cell proliferation and colony forming more effectively than using IDA alone.

In conclusion, these findings suggest that novel vitamin D analogs EB1089 or CB1093 used in combination with IDA may enhance the antileukemic effect. This could either be used to improve the CR rates, or to reduce the dose of cytotoxic medication, thus reducing their adverse effects. On the other hand sequential use of EB1089 or CB 1093 with IDA may not result in enhanced apoptotic effect but may instead abrogate the effects of IDA. Whether this effect is due to the diminished nucleic acid synthesis in differentiated cells requires further study.

7.3 MC1288 is effective in the treatment of both acute and chronic rejection

Several new immunosuppressive molecules have been developed in recent years. The aim has been to find a new immunosuppressive drug with fewer adverse effects than those currently used. The tendency seems to be that by combining drugs, the doses can be lowered and side-effects avoided. A recent candidate is a vitamin D analogue, MC1288, which could provide a new therapeutic approach in organ transplantation.

When we had shown with rat MLC that MC1288 is immunosuppressive in vitro, we tested the drug also in vivo. In our rat aortic allograft model, acute rejection occurs during the first month after transplantation. MC1288 combined with CsA effectively suppressed adventitial inflammatory response by reducing the number of cells and their replication rate in the adventitia of the graft. Our finding of suppression of adventitial inflammatory response in the vascular wall extends earlier studies in which MC1288 prevented acute rejection and prolonged allograft survival in rat heart transplantation, and small bowel transplantation (Johnsson and Tufveson, 1994) .

The number of cells expressing IL-2R was downregulated by MC1288, reflecting a decrease in the number of inflammatory cells in the adventitia, possibly due to inhibition of IL-2R generation, as has been demonstrated earlier (Lemire et al., 1985). As MC1288 binds to the same vitamin D receptor

as native $1\alpha25(OH)_2D_3$, it was expected to bind to activated T cells, thus inhibiting IL-2 production on the transcriptional level (Bhalla et al., 1984; Lemire et al., 1985; Rigby et al., 1984).

The expression of CD4 positive cells was also downregulated, indicating they might be targeted by MC1288. This finding is concordant with earlier results (Lemire et al., 1985; Rigby et al., 1990). On the contrary, the expression of cytotoxic T cells (CD8) was unaltered. This may be expected as $1\alpha,25(OH)_2D_3$ does not affect class I HLA antigen expression on monocytes (Rigby et al., 1990).

The expression of macrophage specific antigen (ED1) was somewhat upregulated in MC1288-treated animals. Rigby et al. have demonstrated that $1\alpha25(OH)_2D_3$ reduces the ability of monocytes to induce antigen-dependent T-cell proliferation. In Rigby´s model the addition of interleukin-1 (IL-1), IL-6, or indomethacin did not restore antigen-dependent T-cell proliferation, suggesting that this observation was not secondary to changes in IL-1, IL-6, or PGE2 production induced by $1\alpha25(OH)_2D_3$. These data suggest that $1\alpha25(OH)_2D_3$ treatment specifically modulates human monocyte phenotype and function, altering HLA-DR antigen expression and antigen presentation, while leaving lytic function intact. (Rigby et al., 1990). This may be an additional pathway that contributes to the inhibition of inflammatory response seen in aortic allografts.

Chronic rejection fully develops three months after transplantation in our model. The typical features, disappearance of smooth muscle cells in media and intimal thickening, were both significantly reduced by either MC1288 or CsA alone or by their combination. This reduction may be due to the relatively high mean concentration of CsA (300-900 ng/ml) during the experiment. It has been demonstrated that there is strong inverse correlation between CsA concentration and development of intimal lesions in rat heart allografts (Koskinen et al., 1995). Thus the novelty of our result was that MC1288 not only prolonged graft survival and prevented acute rejection but also inhibited chronic rejection manifesting as intimal thickening in the vascular wall that ultimately leads to occlusion of vessel and graft loss.

7.4 MC1288 prevents acute graft-versus-host disease in rat bone marrow transplantation

We discovered several new findings with the bone marrow transplant model of our laboratory. First, a novel vitamin D analog, MC1288, either alone or in combination with CsA, prevented clinical signs of GVHD after allogeneic bone marrow transplantation. Second, the histological features of GVHD, i.e. epidermal vacuolar degeneration, portal small lymphocyte infiltration, bile duct injury, edema, fibrosis, and appearance of megakaryocytes in the spleen, were significantly suppressed in MC1288 treated rats. Third, MC1288 effectively suppressed CD4 and CD8 positive T cells, NK cells, MHC class II, IL-2R , and NOS2 expression in the skin and liver, suggesting that MC1288 acts through T and NK cell and macrophage suppression.

Immunopathogenesis of GVHD can be divided into three phases: I) recipient conditioning, II) donor T cell activation, , and III) inflammatory effectors (Krenger et al., 1997). In phase I, host conditioning including total body irradiation or chemotherapy leads to damage and activation of host tissues, which causes increased secretion of inflammatory factors like LPS, tumor necrosis factor α (TNF- α) or interleukin-1 (IL-1) and IL-6.

In phase II, host alloantigens activate donor T cells that differentiate and proliferate into Th1 and/or Th2 type subsets. Specifically, Th1 type cytokine (IFN-γ and IL-2) responses have been postulated to be responsible for GVHD through controlling and amplifying immune cells: CD4+ and CD8+ T cells, NK cells , and monocytes (Krenger et al., 1997). Recently, it has been shown that both Th1 and Th2 type cytokines mediate acute GVHD with distinct end-organ targets (Nikolic et al., 2000). Using STAT4 gene deficient mice that induce Th2 type immune response, as BMT donors, mainly caused liver and skin manifestations of acute GVHD in the recipients. In contrast, intestinal manifestations of GVHD were the major observation in the recipients that received BMT from STAT6 gene deficient mice mounting Th1 type cytokine response. This study clearly demonstrated that both Th1 and Th2 responses are necessary for the development of GVHD. Our study showed that MC1288 effectively decreased the number of CD4+ and CD8+ cells and IL-2 receptor expression in the liver and skin. Whether MC1288 selectively down-regulated

either Th1 or Th2 type cytokine producing cells, or both of them, remains open.

Phase III of acute GVHD culminates in the effector functions that lead to full spectrum of deleterious effects including cachexia and target cell destruction in different organs (Krenger et al., 1997). Mediators of these lesions include inflammatory cytokines and nitric oxide, macrophages/monocytes, NK cells, specific anti-host cytotoxicity mediated by cytotoxic lymphocytes through Fas and perforin apoptotic pathways. We demonstrated that MC1288 effectively decreased both NK cell (NKR-P1A) and NOS2 expression in the liver and skin after BMT suggesting that MC1288 only not influences phase II T cell activation but also suppresses the effector phase.

All in all, the vitamin D analog MC1288 down-regulated both the donor T cell activation (phase II) and inflammatory effector mechanisms (phase III) of acute GVHD after major mismatched allogeneic BMT. MC1288 was even more effective in preventing GVHD when combined with CsA.

7.5 Use of vitamin D analogs as a remission prolonging agent

Low dose maintenance chemotherapy has not been effective in AML and most patients still relapse. Contrary to chemotherapy, differentiating agents are able to induce remission through maturation rather than direct cell kill. This approach could also be effective in eradicating residual leukemia in remission. Residual disease could be eradicated if cells which are resistant to chemotherapy were terminally differentiated resulting to apoptosis. This is suggested by the studies in mice transplanted with syngeneic leukemia cells where vitamin D analogs enhanced the survival of mice with residual leukemia. EB1089 is a very potent inhibitor of leukemic blasts without suppressing the clonal growth of normal hematopoietic progenitor cells (Lee et al., 1996). CB1093 proved to be even more effective in our study. Having achieved remission, the patients could benefit from continuous therapy with the well-tolerated vitamin D analogs. Their antileukemic effect could prevent relapses. Most probably they would be suitable for maintenance therapy after consolidation chemotherapy. Early results suggest, that vitamin D analog could be administered already in the cytopenia stage of recovery, as it has no adverse

effects on hematopoiesis. A phase I study has demonstrated that EB 1089 can be given to AML patients for prolonged periods in oral doses ranging from 5 to 15 µg daily (Pakkala et al. manuscript in preparation).

7.6 Use of vitamin D analogs in immunosuppression

Novel vitamin D analogs may provide new therapeutic options in organ transplantation. MC1288 has been very promising in experimental studies. They are well tolerated, with hypercalcemia being the only major known side effect. Combinations with other immunosuppressants (notably CsA) have been no more hypercalcemic than MC1288 alone, making combination treatment feasible at least in experimental studies.

Vitamin D analogs are especially attractive in stem cell transplantation.
Among the disadvantages of the currently-used pharmacological agents; CsA, prednisone, methotrexate, and FK506 is that they produce generalized immunosuppression and have a negative influence on the beneficial graft-versus-leukemia effect. In contrast to these "traditional" immunosuppressive drugs, vitamin D analogs have also demonstrated anti-leukemic effects in vivo and in vitro. They have no ill effect on hematopoiesis and have in fact been reported to stimulate the proliferation of normal human CD34+ cells (Lee et al., 1996). However, different vitamin D analogs vary significantly in their immunosuppressive, anti-leukemic, and calcemic properties, and therefore further studies are required to assess the most suitable analog for clinical studies.

7.7 Future views

While the research on vitamin D analogs is making progress with promising new analogs and reports regarding various diseases, we still lack the fundamental understanding of the mechanism of action of these agents on a molecular level. By understanding the precise chain of events within the target cell, it would be possible to develop more useful analogs. Instead of synthesizing hundreds of compounds to be meticulously screened, an analog could be modeled specifically with the intended properties. In an ideal situation we would have a very potent differentiating agent with minimal calcemic and immunosuppressive effect to use as a remission-prolonging agent. We would have one potent, well tolerated immunosuppressant with minimal side effects for use in transplantation and in the treatment of autoimmune diseases. The third ideal analog would combine the immunosuppressive with differentiating properties, with minimal side effects to be used in stem cell transplantation for acute leukemia. It would help to eradicate residual leukemia while preventing graft versus host disease.

The understanding of the mechanisms on a molecular level could also provide valuable information regarding the control of the cell cycle, cell maturation and apoptosis.

SUMMARY

The treatment of acute myeloid leukemia would decisively change with a differentiation therapy suitable for clinical use.

The first aim of this study was to investigate the effects of vitamin D analogs on the differentiation and growth of leukemia cells, and combinations of VD_3 analogs with chemotherapy in vitro.

We studied the effects of $1\alpha25(OH)_2D_3$, and two novel vitamin D analogs: EB1089 and CB1093 on HL-60 cells and de novo AML cells in vitro. CB1093 was at least equally effective as the best analogs reported so far in inducing differentiation of HL-60 cells. It was even more effective against de novo AML cells. CB1093 could be better tolerated than most vitamin D analogs due to it´s low calcemic effect. The best antileukemic analog so far has been EB1089 but our study suggested that CB1093 could be given in doses ten times higher than EB1089, at least in rats. This dose would result in antileukemic efficacy 100 times higher than achieved with $1\alpha25(OH)_2D_3$, assuming that the pharmacokinetics are comparable.

We also studied the effect of EB1089 and CB1093 alone, in combination, or in sequence with idarubicin on HL-60 cells. EB1089 and CB1093 were effective in inducing programmed cell death in HL-60 cells. Pretreating the HL-60 cells with vitamin D analogs prior to the exposure to IDA decreased the number of necrotic cells, while simultaneously increasing apoptosis. Combining vitamin D analogs, EB1089 or CB1093 with IDA inhibited HL-60 cell proliferation and colony forming more effectively than using IDA alone.

Stem cell transplantation is common practice in leukemia for patients with a suitable donor. Main problems arise from acute graft-versus-host disease. Due to lack of suitable related donors the use of unrelated, HLA-matched donors from international marrow-donor registries has increased. This has lead to a better prognosis in the treatment of leukemia itself, due to graft-versus-leukemia effect, but with the cost of higher incidence of GVHD.

In solid organ transplantation many transplants are lost due to chronic rejection (transplant arteriosclerosis). The medication used for chronic rejection today has marked side effects that severely limit the immunosuppressive effect that can be induced with these drugs.

The second aim of this study was to investigate vitamin D analogs in the prevention of acute and chronic rejection in a solid organ transplant model, and graft-versus-host disease in a BMT model in vivo.

We initially tested the effect of vitamin D analog MC1288 on lymphocyte proliferation in vitro using mixed lymphocyte cultures. Rat MLC demonstrated that MC1288 was 100-fold more effective than native vitamin D in suppressing stimulated lymphocytes in vitro. To evaluate the effect of MC1288 on preventing acute and chronic rejection in vivo we performed rat aortic allografts that were treated either with MC1288 or cyclosporin A alone or in combination. In our rat aortic allograft model, acute rejection occurs during the first month after transplantation. MC1288 combined with CsA effectively suppressed adventitial inflammatory response by reducing the number of cells and their replication rate in the adventitia of the graft. Chronic rejection develops fully three months after transplantation in our model. The typical features, disappearance of SMC in media and intimal thickening, were both significantly reduced by either MC1288 or CsA alone, or by their combination.

Prevention of graft-versus-host disease was investigated in a major mismatched allogeneic rat bone marrow transplant model. MC1288, either alone or in combination with CsA, prevented clinical signs of GVHD after allogeneic bone marrow transplantation. The histological features of GVHD, i.e. epidermal vacuolar degeneration, portal small lymphocyte infiltration, bile duct injury, edema, fibrosis, and appearance of megakaryocytes in the spleen, were significantly suppressed in MC1288 treated rats. MC1288 effectively suppressed CD4 and CD8 positive T cells, NK cells, MHC class II, IL-2R , and NOS2 expression in the skin and liver, suggesting that MC1288 acts through T cell, NK cell, and macrophage suppression.

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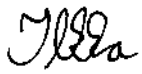

REFERENCES

Abe, E., Miyaura, C., Sakagami, H., Takeda, M., Konno, K., Yamazaki, T., Yoshiki, S., and Suda, T. (1981). Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D3. Proceedings of the National Academy of Sciences of the United States of America *78*, 4990-4.

Aherne, W. A., and Dunnill, M. S. (1982). Morphometry (London: Edward Arnold).

Akutsu, N., Lin, R., Bastien, Y., Bestawros, A., Enepekides, D. J., Black, M. J., and White, J. H. (2001). Regulation of gene Expression by 1alpha,25-dihydroxyvitamin D3 and Its analog EB1089 under growth-inhibitory conditions in squamous carcinoma Cells. Mol Endocrinol *15*, 1127-39.

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature [letter]. Cell *87*, 171 15;102(3):937-43.

Apperley, J. F., Dazzi, F., Craddock, C., and Goldman, J. M. (1998). Immunotherapy for chronic myeloid leukaemia. Hematology & Cell Therapy *40*, 229-32.

Auchincloss, H., Jr., and Sachs, D. H. (1998). Xenogeneic transplantation. Annual Review of Immunology *16*, 433-70.

Bach, F. H., Albertini, R. J., Joo, P., Anderson, J. L., and Bortin, M. M. (1968). Bone-marrow transplantation in a patient with the Wiskott-Aldrich syndrome. Lancet *2*, 1364-6.

Bach, F. H., and Sachs, D. H. (1987). Current concepts: immunology. Transplantation immunology. New England Journal of Medicine *317*, 489-92.

Baker, A. R., McDonnell, D. P., Hughes, M., Crisp, T. M., Mangelsdorf, D. J., Haussler, M. R., Pike, J. W., Shine, J., and O'Malley, B. W. (1988). Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proceedings of the National Academy of Sciences of the United States of America *85*, 3294-8.

Baudet, C., Chevalier, G., Chassevent, A., Canova, C., Filmon, R., Larra, F., Brachet, P., and Wion, D. (1996). 1,25-Dihydroxyvitamin D3 induces programmed cell death in a rat glioma cell line. Journal of Neuroscience Research *46*, 540-50.

Berman, E., Heller, G., Santorsa, J., Mckenzie, S., Gee, T., Kempin, S., Gulati, S., Andreeff, M., Kolitz, J., Gabrilove, J., Reich, L., Mayer, K., Keefe, D., Trainor, K., Schluger, A., Penenberg, D., Raymond, V., O´Reilly, R., Jhanwar, S., Young, C., and Clarkson, B. (1991). Results of a randomized trial comparing idarubicin and cytosine arabinoside with daunorubicin and cytosine arabinoside in adult patients with newly diagnosed acute myelogenous leukemia. Blood *77*, 1666-1674.

Bertolini, D. L., Araujo, P. R., Silva, R. N., Duarte, A. J., and Tzanno-Martins, C. B. (1999). Immunomodulatory effects of vitamin D analog KH1060 on an experimental skin transplantation model. Transplant Proc *31*, 2998-9.

Bhalla, A., Amento, E., Clemens, T., Holick, M., and Krane, S. (1983). Specific high-affinity receptors for 1, 25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: Presence in monocytes and induction in T lymphocytes following activation. J Clin Endocrinol Metab *57*, 1308-1310.

Bhalla, A., Amento, E., Serog, B., and Glimcher, L. (1984). 1,25-Dihydroxyvitamin D3 inhibits antigen-induced T cell activation. J Immunol *133*, 1748-1754.

Binderup, L., Latini, S., Binderup, E., Brertting, C., Calverley, M., and Hansen, K. (1991). 20-epi-vitamin D3 analogues: a novel class of potent regulators of cell growth and immune response. Biochem Pharmacol *42*, 1569-1575.

Bodmer, J. G., Marsh, S. G., Albert, E. D., Bodmer, W. F., Bontrop, R. E., Dupont, B., Erlich, H. A., Hansen, J. A., Mach, B., Mayr, W. R., Parham, P., Petersdorf, E. W., Sasazuki, T., Schreuder, G. M., Strominger, J. L., Svejgaard, A., and Terasaki, P. I. (1999). Nomenclature for factors of the HLA system, 1998. Tissue Antigens *53*, 407-46.

Boehler, A., and Estenne, M. (2000). Obliterative bronchiolitis after lung transplantation. Current Opinion in Pulmonary Medicine *6*, 133-9.

Breitman, T. R., Selonick, S. E., and Collins, S. J. (1980). Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proceedings of the National Academy of Sciences of the United States of America *77*, 2936-40.

Brenner, R. V., Shabahang, M., Schumaker, L. M., Nauta, R. J., Uskokovic, M. R., Evans, S. R., and Buras, R. R. (1995). The antiproliferative effect of vitamin D analogs on MCF-7 human breast cancer cells. Cancer Letters *92*, 77-82.

Brown, R. A., Adkins, D., Khoury, H., Vij, R., Goodnough, L. T., Shenoy, S., and DiPersio, J. F. (1999). Long-term follow-up of high-risk allogeneic peripheral-blood stem-cell transplant recipients: graft-versus-host disease and transplant-related mortality. Journal of Clinical Oncology *17*, 806-12.

Bugge, T. H., Pohl, J., Lonnoy, O., and Stunnenberg, H. G. (1992). RXR alpha, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO Journal *11*, 1409-18.

Campbell, P., and Halloran, P. (1996). Antibody-mediated rejection. In Solid organ transplant rejection. Mechanisms, pathology, and diagnosis., K. Solez, L. Racusen and M. Billingham, eds. (New York: Marcel Dekker Inc), pp. 29-58.

Castaigne, S., Chomienne, C., Daniel, M., Ballerini, P., Berger, R., Fenaux, P., and Degos, L. (1990). All-trans retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. Blood *76*, 1704-1709.

Casteels, K., Waer, M., Laureys, J., Valckx, D., Depovere, J., Bouillon, R., and Mathieu, C. (1998). Prevention of autoimmune destruction of syngeneic islet grafts in spontaneously diabetic nonobese diabetic mice by a combination of a vitamin D3 analog and cyclosporine. Transplantation *65*, 1225-32.

Chaudhry, M., Sundaram, S., Gennings, C., Carter, H., and Gewirtz, D. A. (2001). The vitamin D3 analog, ILX-23-7553, enhances the response to adriamycin and irradiation in MCF-7 breast tumor cells. Cancer Chemother Pharmacol *47*, 429-36.

Chen, Z., Xue, Y., Zhang, R., Tao, R., Xia, X., Li, C., Wang, W., Zu, W., Yao, X., and Ling, B. (1991). A clinical and experimental study on all-trans retinoic acid-treated acute promyelocytic leukemia patients. Blood *78*, 1413-1419.

Collins, S. (1987). The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood *70*, 1233-1244.

Collins, S., Gallo, R., and Gallagher, R. (1977). Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. Nature *270*, 347-349.

Colston, K., Colston, M. J., and Feldman, D. (1981). 1,25-dihydroxyvitamin D3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. Endocrinology *108*, 1083-6.

Colston, K. W., Chander, S. K., Mackay, A. G., and Coombes, R. C. (1992). Effects of synthetic vitamin D analogues on breast cancer cell proliferation in vivo and in vitro. Biochemical Pharmacology *44*, 693-702.

Danielsson, C., Fehsel, K., Polly, P., and Carlberg, C. (1998). Differential apoptotic response of human melanoma cells to 1 alpha,25-dihydroxyvitamin D3 and its analogues. Cell Death & Differentiation *5*, 946-52.

Date, H. (2001). Current status and future of lung transplantation. Internal Medicine *40*, 87-95.

Diaz, G. D., Paraskeva, C., Thomas, M. G., Binderup, L., and Hague, A. (2000). Apoptosis is induced by the active metabolite of vitamin D3 and its analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy. Cancer Research *60*, 2304-12.

Doyle, C., and Strominger, J. L. (1987). Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature *330*, 256-9.

Eisman, J. A., Martin, T. J., MacIntyre, I., and Moseley, J. M. (1979). 1,25-dihydroxyvitamin-D-receptor in breast cancer cells. Lancet *2*, 1335-6.

Elssner, A., and Vogelmeier, C. (2001). The role of neutrophils in the pathogenesis of obliterative bronchiolitis after lung transplantation. Transplant Infectious Disease *3*, 168-76.

Elstner, E., Linker-Israeli, M., Umiel, T., Le, J., Grillier, I., Said, J., Shintaku, I. P., Krajewski, S., Reed, J. C., Binderup, L., and Koeffler, H. P. (1996). Combination

of a potent 20-epi-vitamin D3 analogue (KH 1060) with 9-cis-retinoic acid irreversibly inhibits clonal growth, decreases bcl-2 expression, and induces apoptosis in HL-60 leukemic cells. Cancer Research *56*, 3570-6.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889-95.

Evans, S. R., Soldatenkov, V., Shchepotin, E. B., Bogrash, E., and Shchepotin, I. B. (1999). Novel 19-nor-hexafluoride vitamin D3 analog (Ro 25-6760) inhibits human colon cancer in vitro via apoptosis. Int J Oncol *14*, 979-85.

Feldman, D., Chen, T., Hirst, M., Colston, K., Karasek, M., and Cone, C. (1980). Demonstration of 1,25-dihydroxyvitamin D3 receptors in human skin biopsies. Journal of Clinical Endocrinology & Metabolism *51*, 1463-5.

Ferrara, J. L., and Deeg, H. J. (1991). Graft-versus-host disease. New England Journal of Medicine *324*, 667-74.

Fibach, E., Landau, T., and Sachs, L. (1972). Normal differentiation of myeloid leukaemic cells induced by a differentiation-inducing protein. Nature - New Biology *237*, 276-8.

Fife, R. S., Sledge, G. W., Jr., and Proctor, C. (1997). Effects of vitamin D3 on proliferation of cancer cells in vitro. Cancer Letters *120*, 65-9.

Goldman, J. M., Gale, R. P., Horowitz, M. M., Biggs, J. C., Champlin, R. E., Gluckman, E., Hoffmann, R. G., Jacobsen, S. J., Marmont, A. M., McGlave, P. B., and et al. (1988). Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion. Annals of Internal Medicine *108*, 806-14.

Goldman, J. M., Schmitz, N., Niethammer, D., and Gratwohl, A. (1998). Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe in 1998. Accreditation Sub-Committee of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplantation *21*, 1-7.

Gratwohl, A., Passweg, J., Baldomero, H., Hermans, J., and Urbano-Ispizua, A. (2000). Hematopoietic stem cell transplantation in Europe 1998. The Hematology Journal *1*, 333-350.

Green, D. R., and Reed, J. C. (1998). Mitochondria and apoptosis. Science *281*, 1309-12.

Grillier, I., Umiel, T., Elstner, E., Collins, S. J., and Koeffler, H. P. (1997). Alterations of differentiation, clonal proliferation, cell cycle progression and bcl-2 expression in RAR alpha-altered sublines of HL-60. Leukemia *11*, 393-400.

Hall, B., and Suranyi, M. (1995). Understanding rejection. In The Handbook of Transplant Immunology, K. Wood and P. Morris, eds.: MedSci Publications), pp. 113-133.

Hernandez-Fuentes, M. P., Baker, R. J., and Lechler, R. I. (1999). The alloresponse. Reviews in Immunogenetics *1*, 282-96.

Hershberger, P. A., Modzelewski, R. A., Shurin, Z. R., Rueger, R. M., Trump, D. L., and Johnson, C. S. (1999). 1,25-Dihydroxycholecalciferol (1,25-D3) inhibits the growth of squamous cell carcinoma and down-modulates p21(Waf1/Cip1) in vitro and in vivo. Cancer Research *59*, 2644-9.

Hisatake, J., O'Kelly, J., Uskokovic, M. R., Tomoyasu, S., and Koeffler, H. P. (2001). Novel vitamin D(3) analog, 21-(3-methyl-3-hydroxy-butyl)-19-nor D(3), that modulates cell growth, differentiation, apoptosis, cell cycle, and induction of PTEN in leukemic cells. Blood *97*, 2427-33.

Honma, Y., Hozumi, M., Abe, E., Konno, K., Fukushima, M., Hata, S., Nishii, Y., DeLuca, H., and Suda, T. (1983). 1,25-dihydroxyvitamin D3 and 1 alpha-hydroxyvitamin D3 prolongs survival time of mice inoculated with myeloid leukemia cells. Proc. Natl. Acad. Sci. USA *80*, 201-204.

Huang, M., Ye, Y., Chen, S., Chai, J., Lu, J., Zhoa, L., Gu, L., and Wang, Z. (1988). Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood *72*, 567-572.

James, K. B., Bott-Silverman, C., Houghtaling, P., Ratliff, N., Magyar, W., and Nissen, S. (2001). Contribution of vascular rejection to progression of transplant coronary disease as assessed by serial intravascular ultrasound. American Journal of Cardiology *88*, 707-10.

James, S. Y., Mackay, A. G., Binderup, L., and Colston, K. W. (1994). Effects of a new synthetic vitamin D analogue, EB1089, on the oestrogen-responsive growth of human breast cancer cells. Journal of Endocrinology *141*, 555-63.

James, S. Y., Mercer, E., Brady, M., Binderup, L., and Colston, K. W. (1998). EB1089, a synthetic analogue of vitamin D, induces apoptosis in breast cancer cells in vivo and in vitro. British Journal of Pharmacology *125*, 953-62.

Jiang, H., Lin, J., Su, Z. Z., Collart, F. R., Huberman, E., and Fisher, P. B. (1994). Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene *9*, 3397-406.

Johnsson, C., and Tufveson, G. (1994). MC1288-A vitamin D analogue with immunosuppressive effects on heart and small bowel grafts. Transplant Int *7*, 392-397.

Johnsson, C., and Tufveson, G. (1994). MC 1288--a vitamin D analogue with immunosuppressive effects on heart and small bowel grafts. Transplant International *7*, 392-7.

Ketley, N. J., Allen, P. D., Kelsey, S. M., and Newland, A. C. (1997). Modulation of idarubicin-induced apoptosis in human acute myeloid leukemia blasts by all-trans retinoic acid, 1,25(OH)2 vitamin D3, and granulocyte-macrophage colony-stimulating factor. Blood *90*, 4578-87.

Kliewer, S. A., Umesono, K., Mangelsdorf, D. J., and Evans, R. M. (1992). Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. Nature *355*, 446-9.

Koeffler, H., Hirji, K., Itri, L., and Group, S. C. L. (1985). 1,25-Dihydroxyvitamin D3: in vivo and in vitro effects on human preleukemic and leukemic cells. Cancer Treat. Rep. *69*, 1399-1407.

Koeffler, H. P. (1983). Induction of differentiation of human acute myelogenous leukemia cells: therapeutic implications. Blood *62*, 709-21.

Koskinen, P., Lemström, K., and Häyry, P. (1995). How cyclosporine modifies histological and molecular events in the vascular wall during chronic rejection of rat cardiac allografts. Am J Pathol *146*, 972-980.

Krenger, W., Hill, G. R., and Ferrara, J. L. (1997). Cytokine cascades in acute graft-versus-host disease. Transplantation *64*, 553-8.

Lee, Y. Y., Kim, E. S., Seol, J. G., Kim, B. K., Binderup, L., Elstner, E., Park, D. J., and Koeffler, H. P. (1996). Effect of a vitamin D3 analog, EB1089, on hematopoietic stem cells from normal and myeloid leukemic blasts. Leukemia *10*, 1751-7.

Lemire, J., Adams, J., Kermani-Arab, V., Bakke, A., Sakai, R., and Jordan, S. (1985). 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity in vitro. J Immunol *134*, 3032-3035.

Lemire, J., and Archer, D. (1991). 1,25-Dihydroxyvitamin D3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. J Clin Invest *87*, 1103-1107.

Lemire, J., Ince, A., and Takashima, M. (1992). 1,25-Dihydroxyvitamin D3 attenuates the expression of experimental murine lupus of MRL/l mice. Autoimmunity *12*, 143-148.

Lemire, J. M., Archer, D. C., Khulkarni, A., Ince, A., Uskokovic, M. R., and Stepkowski, S. (1992). Prolongation of the survival of murine cardiac allografts by the vitamin D3 analogue 1,25-dihydroxy-delta 16-cholecalciferol. Transplantation *54*, 762-3.

Lerner, K. G., Kao, G. F., Storb, R., Buckner, C. D., Clift, R. A., and Thomas, E. D. (1974). Histopathology of graft-vs.-host reaction (GvHR) in human recipients of marrow from HL-A-matched sibling donors. Transplantation Proceedings *6*, 367-71.

Lillevang, S., Rosenkvist, J., Andersen, C., Larsen, S., Kemp, E., and Kristensen, T. (1992). Single and combined effects of the vitamin D analogue KH1060 and Cyclosporin A on mercuric-chloride-induced autoimmune disease in the BN rat. Clin Exp Immunol *88*, 301-306.

Linsley, P. S., Clark, E. A., and Ledbetter, J. A. (1990). T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1.

Proceedings of the National Academy of Sciences of the United States of America *87*, 5031-5.

Liszewski, M. K., Farries, T. C., Lublin, D. M., Rooney, I. A., and Atkinson, J. P. (1996). Control of the complement system. Advances in Immunology *61*, 201-83.

Liu, M., Lee, M. H., Cohen, M., Bommakanti, M., and Freedman, L. P. (1996). Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes & Development *10*, 142-53.

Lowes, J. R., Hubscher, S. G., and Neuberger, J. M. (1993). Chronic rejection of the liver allograft. Gastroenterology Clinics of North America *22*, 401-20.

Mach, B. (1994). Genetics of histocompatibility. Current Opinion in Hematology *1*, 4-11.

Majewski, S., Kutner, A., and Jablonska, S. (2000). Vitamin D analogs in cutaneous malignancies. Current Pharmaceutical Design *6*, 829-38.

Mathiasen, I. S., Lademann, U., and Jaattela, M. (1999). Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. Cancer Research *59*, 4848-56.

Mennander, A., Tiisala, S., Halttunen, J., Yilmaz, S., Paavonen, T., and Hayry, P. (1991). Chronic rejection in rat aortic allografts. An experimental model for transplant arteriosclerosis. Arterioscler Thromb *11*, 671-80.

Mihatsch, M. J., Kyo, M., Morozumi, K., Yamaguchi, Y., Nickeleit, V., and Ryffel, B. (1998). The side-effects of ciclosporine-A and tacrolimus. Clinical Nephrology *49*, 356-63.

Miyaura, C., Abe, E., Kuribayashi, T., Tanaka, H., Konno, K., Nishii, Y., and Suda, T. (1981). 1 alpha,25-Dihydroxyvitamin D3 induces differentiation of human myeloid leukemia cells. Biochemical & Biophysical Research Communications *102*, 937-43.

Montixi, C., Langlet, C., Bernard, A. M., Thimonier, J., Dubois, C., Wurbel, M. A., Chauvin, J. P., Pierres, M., and He, H. T. (1998). Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. Embo J *17*, 5334-48.

Muto, A., Kizaki, M., Yamato, K., Kawai, Y., Kamata-Matsushita, M., Ueno, H., Ohguchi, M., Nishihara, T., Koeffler, H. P., and Ikeda, Y. (1999). 1,25-Dihydroxyvitamin D3 induces differentiation of a retinoic acid-resistant acute promyelocytic leukemia cell line (UF-1) associated with expression of p21(WAF1/CIP1) and p27(KIP1). Blood *93*, 2225-33.

Naveilhan, P., Berger, F., Haddad, K., Barbot, N., Benabid, A. L., Brachet, P., and Wion, D. (1994). Induction of glioma cell death by 1,25(OH)2 vitamin D3: towards an endocrine therapy of brain tumors? Journal of Neuroscience Research *37*, 271-7.

Nikolic, B., Lee, S., Bronson, R., Grusby, M., and Sykes, M. (2000). Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. J Clin Invest *105*, 1289-1298.

Norman, A. W., Roth, J., and Orci, L. (1982). The vitamin D endocrine system: steroid metabolism, hormone receptors, and biological response (calcium binding proteins). Endocrine Reviews *3*, 331-66.

Paavonen, T., Mennander, A., Lautenschlager, I., Mattila, S., and Hayry, P. (1993). Endothelialitis and accelerated arteriosclerosis in human heart transplant coronaries. Journal of Heart & Lung Transplantation *12*, 117-22.

Pakkala, S., de Vos, S., Elstner, E., Rude, R. K., Uskokovic, M., Binderup, L., and Koeffler, H. P. (1995). Vitamin D3 analogs: effect on leukemic clonal growth and differentiation, and on serum calcium levels. Leukemia Research *19*, 65-72.

Park, W. H., Seol, J. G., Kim, E. S., Hyun, J. M., Jung, C. W., Lee, C. C., Binderup, L., Koeffler, H. P., Kim, B. K., and Lee, Y. Y. (2000). Induction of apoptosis by vitamin D3 analogue EB1089 in NCI-H929 myeloma cells via activation of caspase 3 and p38 MAP kinase. British Journal of Haematology *109*, 576-83.

Paterson, P. Y. (1976). Experimental autoimmune encephalomyelitis: induction, pathogenesis and suppression. In Textbook of Immunopathology, P. A. Miescher and H. J. Muller-Eberhard, eds. (New York: Grune and Stratton Inc), pp. 179-213.

Peehl, D. M., Skowronski, R. J., Leung, G. K., Wong, S. T., Stamey, T. A., and Feldman, D. (1994). Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. Cancer Research *54*, 805-10.

Peetre, C., Gullberg, U., Nilsson, E., and Olsson, I. (1986). Effects of recombinant tumor necrosis factor on proliferation and differentiation of leukemic and normal hemopoietic cells in vitro. Relationship to cell surface receptor. Journal of Clinical Investigation *78*, 1694-700.

Petkovich, M., Brand, N. J., Krust, A., and Chambon, P. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature *330*, 444-50.

Phillips, G., Reece, D., Shepherd, J., Barnett, M., Brown, R., Frei-Lahr, D., Klingemann, H., Bolwell, B., Spinelli, J., Herzig, R., and Herzig, G. (1991). High-dose cytarabine and daunorubicin induction and postremission chemotherapy for the treatment of acute myelogenous leukemia in adults. Blood *77*, 1429-1435.

Ponticelli, C. (2000). Progression of renal damage in chronic rejection. Kidney International - Supplement *75*, S62-70.

Provvedini, D., Tsoukas, C., Deftos, L., and Manolagos, S. (1986). 1a, 25-Dihydroxyvitamin D3-binding macromolecules in human B lymphocytes: Effects on immunoglobulin production. J Immunol *136*, 2734-2740.

Reed, J. C. (1998). Bcl-2 family proteins. Oncogene *17*, 3225-36.

Reed, J. C. (1999). Caspases and cytokines: roles in inflammation and autoimmunity. Advances in Immunology *73*, 265-99.

Reed, J. C. (2000). Mechanisms of apoptosis. American Journal of Pathology *157*, 1415-30.

Reed, J. C., Miyashita, T., Takayama, S., Wang, H. G., Sato, T., Krajewski, S., Aime-Sempe, C., Bodrug, S., Kitada, S., and Hanada, M. (1996). BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. J Cell Biochem *60*, 23-32.

Reichel, H., Koeffler, H., Tobler, A., and Norman, A. (1987). 1a,25-Dihydroxyvitamin D3 inhibits g-interferon synthesis by normal human peripheral blood lymphocytes. Proc Natl Acad Sci USA *84*, 3385-3389.

Renkonen, R., and Häyry, P. (1984). Bone marrow transplantation in the rat. I. Histologic correlations and quantitation of cellular infiltrates in acute graft-versus-host disease. American Journal of Pathology *117*, 462-70.

Rigby, W., Stacy, T., and Fanger, M. (1984). Inhibition of T lymphocyte mitogenesis by 1,25-Dihydroxyvitamin D3 (Calcitriol). J Clin Invest *74*, 1451-1455.

Rigby, W., Waugh, M., and Graziano, R. (1990). Regulation of human monocyte HLA-DR and CD4 antigen expression, and antigen presentation by 1,25-Dihydroxyvitamin D3. Blood *76*, 189-197.

Robson, S. C., Schulte am Esch, J., 2nd, and Bach, F. H. (1999). Factors in xenograft rejection. Annals of the New York Academy of Sciences *875*, 261-76.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature *362*, 801-9.

Sachs, L. (1995). The adventures of a biologist: prenatal diagnosis, hematopoiesis, leukemia, carcinogenesis, and tumor suppression. Advances in Cancer Research *66*, 1-40.

Sachs, L. (1990). The control of growth and differentiation in normal and leukemic blood cells. Cancer *65*, 2196-206.

Sachs, L. (1978). Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukaemia. Nature *274*, 535-9.

Sachs, L. (1987). The molecular control of blood cell development. Science *238*, 1374-9.

Sale, G. E., Lerner, K. G., Barker, E. A., Shulman, H. M., and Thomas, E. D. (1977). The skin biopsy in the diagnosis of acute graft-versus-host disease in man. American Journal of Pathology *89*, 621-35.

Salvesen, G. S., and Dixit, V. M. (1997). Caspases: intracellular signaling by proteolysis. Cell *91*, 443-6.

Saunders, D. E., Christensen, C., Williams, J. R., Wappler, N. L., Lawrence, W. D., Malone, J. M., Malviya, V. K., and Deppe, G. (1995). Inhibition of breast and ovarian carcinoma cell growth by 1,25-dihydroxyvitamin D3 combined with retinoic acid or dexamethasone. Anti-Cancer Drugs *6*, 562-9.

Sayegh, M. H., and Turka, L. A. (1998). The role of T-cell costimulatory activation pathways in transplant rejection. New England Journal of Medicine *338*, 1813-21.

Schiffer, C. A., Dodge, R., and Larson, R. A. (1997). Long-term follow-up of Cancer and Leukemia Group B studies in acute myeloid leukemia. Cancer *80*, 2210-4.

Schwaller, J., Koeffler, H. P., Niklaus, G., Loetscher, P., Nagel, S., Fey, M. F., and Tobler, A. (1995). Posttranscriptional stabilization underlies p53-independent induction of p21WAF1/CIP1/SDI1 in differentiating human leukemic cells. Journal of Clinical Investigation *95*, 973-9.

Shabahang, M., Buras, R. R., Davoodi, F., Schumaker, L. M., Nauta, R. J., Uskokovic, M. R., Brenner, R. V., and Evans, S. R. (1994). Growth inhibition of HT-29 human colon cancer cells by analogues of 1,25-dihydroxyvitamin D3. Cancer Research *54*, 4057-64.

Shany, S., Levy, Y., and Lahav-Cohen, M. (2001). The effects of 1alpha,24(S)-dihydroxyvitamin D(2) analog on cancer cell proliferation and cytokine expression. Steroids *66*, 319-25.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569-72.

Skowronski, R. J., Peehl, D. M., and Feldman, D. (1995). Actions of vitamin D3, analogs on human prostate cancer cell lines: comparison with 1,25-dihydroxyvitamin D3. Endocrinology *136*, 20-6.

Slavin, R. E., and Santos, G. W. (1973). The graft versus host reaction in man after bone marrow transplantation: pathology, pathogenesis, clinical features, and implication. Clinical Immunology & Immunopathology *1*, 472-98.

Solez, K., Vincenti, F., and Filo, R. S. (1998). Histopathologic findings from 2-year protocol biopsies from a U.S. multicenter kidney transplant trial comparing tarolimus versus cyclosporine: a report of the FK506 Kidney Transplant Study Group. Transplantation *66*, 1736-40.

Sundaram, S., Chaudhry, M., Reardon, D., Gupta, M., and Gewirtz, D. A. (2000). The vitamin D3 analog EB 1089 enhances the antiproliferative and apoptotic effects of adriamycin in MCF-7 breast tumor cells. Breast Cancer Res Treat *63*, 1-10.

Tanaka, H., Abe, E., Miyaura, C., Kuribayashi, T., Konno, K., Nishii, Y., and Suda, T. (1982). 1 alpha,25-Dihydroxycholecalciferol and a human myeloid leukaemia cell line (HL-60). Biochemical Journal *204*, 713-9.

Tsoukas, C. D., Provvedini, D. M., and Manolagas, S. C. (1984). 1,25-dihydroxyvitamin D3: a novel immunoregulatory hormone. Science *224*, 1438-40.

Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C. M. (1985). Involvement of the bcl-2 gene in human follicular lymphoma. Science *228*, 1440-3.

Tyndall, A., and Gratwohl, A. (1997). Hemopoietic blood and marrow transplants in the treatment of severe autoimmune disease. Current Opinion in Hematology *4*, 390-4.

van Etten, E., Gysemans, C., Verstuyf, A., Bouillon, R., and Mathieu, C. (2001). Immunomodulatory properties of a 1,25(OH)(2) vitamin D(3) analog combined with IFNbeta in an animal model of syngeneic islet transplantation. Transplant Proc *33*, 2319.

van Rhee, F., Szydlo, R. M., Hermans, J., Devergie, A., Frassoni, F., Arcese, W., de Witte, T., Kolb, H. J., Niederwiser, D., Jacobsen, N., Gahrton, G., Bandini, G., Carreras, E., Bacigalupo, A., Michallet, M., Ruutu, T., Reiffers, J., Goldman, J. M., Apperley, J., and Gratwohl, A. (1997). Long-term results after allogeneic bone marrow transplantation for chronic myelogenous leukemia in chronic phase: a report from the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplantation *20*, 553-60.

Veyron, P., Pamphile, R., Binderup, L., and Touraine, J.-L. (1993). Two novel vitamin D analogues, KH1060 and CB966, prolong skin allograft survival in mice. Transplant Immunol *1*, 72-76.

Viola, A., and Lanzavecchia, A. (1996). T cell activation determined by T cell receptor number and tunable thresholds. Science *273*, 104-6.

Wali, R. K., Bissonnette, M., Khare, S., Hart, J., Sitrin, M. D., and Brasitus, T. A. (1995). 1 alpha,25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol, a noncalcemic analogue of 1 alpha,25-dihydroxyvitamin D3, inhibits azoxymethane-induced colonic tumorigenesis. Cancer Research *55*, 3050-4.

Wallach, D., Varfolomeev, E. E., Malinin, N. L., Goltsev, Y. V., Kovalenko, A. V., and Boldin, M. P. (1999). Tumor necrosis factor receptor and Fas signaling mechanisms. Annual Review of Immunology *17*, 331-67.

Wang, Q. M., Jones, J. B., and Studzinski, G. P. (1996). Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. Cancer Research *56*, 264-7.

Warrell, R. J., Frankell, S., Miller, W., Scheinberg, D., Itri, L., Hittelman, W., Vyas, R., Andreeff, M., Tafuri, A., Jakubowski, A., Gabrilove, J., Gordon, M., and Dmitrinovsky, E. (1991). Differentiation therapy of acute promyelocyti

leukemia with tretinoin (all-trans-retinoic acid). New Engl. J. Med. *324*, 1385-1393.

Xavier, R., Brennan, T., Li, Q., McCormack, C., and Seed, B. (1998). Membrane compartmentation is required for efficient T cell activation. Immunity *8*, 723-32.

Yang, E., and Korsmeyer, S. J. (1996). Molecular thanatopsis: a discourse on the BCL2 family and cell death. Blood *88*, 386-401.

Yang, L., Yang, J., Venkateswarlu, S., Ko, T., and Brattain, M. G. (2001). Autocrine TGFbeta signaling mediates vitamin D3 analog-induced growth inhibition in breast cells. J Cell Physiol *188*, 383-93.

Yin, C., Knudson, C. M., Korsmeyer, S. J., and Van Dyke, T. (1997). Bax suppresses tumorigenesis and stimulates apoptosis in vivo. Nature *385*, 637-40.

Yu, J., Papavasiliou, V., Rhim, J., Goltzman, D., and Kremer, R. (1995). Vitamin D analogs: new therapeutic agents for the treatment of squamous cancer and its associated hypercalcemia. Anti-Cancer Drugs *6*, 101-8.

Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K., and Rosenfeld, M. G. (1991). RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell *67*, 1251-66.

Yu, Y., Kumar, V., and Bennett, M. (1992). Murine Natural killer cells and marrow graft rejection. Annu Rev Immunol *10*, 189-214.

Yuan, J. (1997). Transducing signals of life and death. Current Opinion in Cell Biology *9*, 247-51.

Zhou, J., Norman, A., Chen, D., Sun, G., Uskokovic, M., and Koeffler, H. (1990). 1,25-dihydroxy-16-ene-23-yne-vitamin D3 prolongs survival time of leukemic mice. Proc. Natl. Acad. Sci. USA *87*, 3929-3932.

Zhou, J., Norman, A., Lübbert, M., Collins, E., Uskokovic, M., and Koeffler, H. (1989). Novel vitamin D analogs that modulate leukemic cell growth and differentiation with little effect on either intestinal calcium absorption or bone calcium mobilization. Blood *74*, 82-93.

Zittoun, R., Jehn, U., Fiere, D., Haanen, C., Löwenberg, B., Willemze, R., Abels, J., Bury, J., Peetermans, M., Hayat, M., Suciu, S., Solbu, G., and Stryckmans, P. (1989). Alternating v repeated postremission treatment in adult acute myelogenous leukemia: A randomized Phase III study (AML6) of the EORTC leukemia cooperative group. Blood *73*, 896-906.